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in its capacity as elected Office

Date of mailing (day/month/year) 27 March 2001 (27.03.01)	
International application No. PCT/IB00/01026	**Applicant's or agent's file reference** P022260WO
International filing date (day/month/year) 13 July 2000 (13.07.00)	**Priority date** (day/month/year) 14 July 1999 (14.07.99)
Applicant MASIGNANI, Vega et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

14 February 2001 (14.02.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Lazar Joseph Panakal
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 January 2001 (18.01.2001)

PCT

(10) International Publication Number
WO 01/04316 A2

(51) International Patent Classification[7]: C12N 15/31, C07K 14/22, G01N 33/53, C12Q 1/68, C07K 16/12, A61K 39/095, 39/395, 48/00

(21) International Application Number: PCT/IB00/01026

(22) International Filing Date: 13 July 2000 (13.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9916529.2 14 July 1999 (14.07.1999) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/04316 A2

(54) Title: ANTIGENIC MENINGOCOCCAL PEPTIDES

(57) Abstract: WO99/36544 discloses a large number of proteins from *Neisseria Meningitidis*. The present invention relates to fragments of those proteins which comprise at least one antigenic determinant. Homologous sequences and proteins comprising these fragments are also disclosed.

ANTIGENIC MENINGOCOCCAL PEPTIDES

All documents cited herein are incorporated by reference in their entirety. In particular, the contents of international patent application WO99/36544 are fully incorporated herein.

FIELD OF THE INVENTION

This invention relates to antigenic peptide sequences from the bacterium *Neisseria meningitidis*.

BACKGROUND

Neisseria meningitidis is a non-motile, Gram-negative diplococcus that is pathogenic in humans.

Based on the organism's capsular polysaccharide, 12 serogroups of *N.meningitidis* have been identified. Group A is the pathogen most often implicated in epidemic disease in sub-Saharan Africa. Serogroups B and C are responsible for the vast majority of cases in the United States and in most developed countries. Serogroups W135 and Y are responsible for the rest of the cases in the United States and developed countries.

The meningococcal vaccine currently in use is a tetravalent polysaccharide vaccine composed of serogroups A, C, Y and W135. Meningococcus B remains a problem, however. The polysaccharide approach cannot be used because the menB capsular polysaccharide is a polymer of α(2-8)-linked *N*-acetyl neuraminic acid that is also present in mammalian tissue. One approach to a menB vaccine uses mixtures of outer membrane proteins (OMPs) To overcome the antigenic variability, multivalent vaccines containing up to nine different porins have been constructed [*e.g.* Poolman JT (1992) *Infect. Agents Dis.* 4:13-28]. Additional proteins to be used in outer membrane vaccines have been the opa and opc proteins, but none of these approaches have been able to overcome the antigenic variability [*e.g.* Ala'Aldeen & Borriello (1996) *Vaccine* 14(1):49-53].

THE INVENTION

The invention provides fragments of the proteins disclosed in international patent application WO99/36544, wherein the fragments comprise at least one antigenic determinant.

Thus, if the length of any particular protein sequence disclosed in WO99/36544 is *x* amino acids (see Table II), the present invention provides fragments of at most *x-1* amino acids of that protein. The fragment may be shorter than this (*e.g. x-2, x-3, x-4, ...*), and is preferably 100 amino acids or less (*e.g.* 90 amino acids, 80 amino acids *etc.*). The fragment may be as short as 3

amino acids, but is preferably longer (*e.g.* up to 6, 7, 8, 9, 10, 12, 15, 20, 25, 30, 35, 40, 50, 75, or 100 amino acids).

Preferred fragments comprise the meningococcal peptide sequences disclosed in Table I, or sub-sequences thereof. The fragments may be longer than those given in Table I *e.g.* where a fragment in Table I runs from amino acid residue *p* to residue *q* of a protein, the invention also relates to fragments from residue (*p-1*), (*p-2*), or (*p-3*) to residue (*q+1*), (*q+2*), or (*q+3*).

The invention also provides polypeptides that are homologous (*i.e.* have sequence identity) to these fragments. Depending on the particular fragment, the degree of sequence identity is preferably greater than 50% (*e.g.* 60%, 70%, 80%, 90%, 95%, 99% or more). These homologous polypeptides include mutants and allelic variants of the fragments. Identity between the two sequences is preferably determined by the Smith-Waterman homology search algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters *gap open penalty*=12 and *gap extension penalty*=1.

The invention also provides proteins comprising one or more of the above-defined fragments.

The invention is subject to the proviso that it does not include within its scope proteins comprising any of the 45 protein sequences disclosed in WO99/36544 (*i.e.* the even SEQ IDs: 2, 4, 6, 8, 10, ..., 86, 88, 90 of WO99/36544).

The proteins of the invention can, of course, be prepared by various means (*e.g.* recombinant expression, purification from cell culture, chemical synthesis *etc.*) and in various forms (*e.g.* native, C-terminal and/or N-terminal fusions *etc.*). They are preferably prepared in substantially pure form (*i.e.* substantially free from other Neisserial or host cell proteins). Short proteins are preferably produced using chemical peptide synthesis.

According to a further aspect, the invention provides antibodies which recognise the fragments of the invention, with the proviso that the invention does not include within its scope antibodies which recognise one of 45 complete protein sequences in WO99/36544. The antibodies may be polyclonal or, preferably, monoclonal, and may be produced by any suitable means.

The invention also provides proteins comprising peptide sequences recognised by these antibodies. These peptide sequences will, of course, include fragments of the meningococcal proteins in WO99/36544, but will also include peptides that mimic the antigenic structure of the meningococcal peptides when bound to immunoglobulin.

According to a further aspect, the invention provides nucleic acid encoding the fragments and proteins of the invention, with the proviso that the invention does not include within its scope nucleic acid encoding one of the 45 protein sequences in WO99/36544.

In addition, the invention provides nucleic acid comprising sequences homologous (*i.e.* having sequence identity) to these sequences. Furthermore, the invention provides nucleic acid which can hybridise to these sequences, preferably under "high stringency" conditions (*e.g.* 65°C in a 0.1xSSC, 0.5% SDS solution).

It should also be appreciated that the invention provides nucleic acid comprising sequences complementary to those described above (*e.g.* for antisense or probing purposes).

Nucleic acid according to the invention can, of course, be prepared in many ways (*e.g.* by chemical synthesis, from genomic or cDNA libraries, from the organism itself *etc.*) and can take various forms (*e.g.* single stranded, double stranded, vectors, probes *etc.*). In addition, the term "nucleic acid" includes DNA and RNA, and also their analogues, such as those containing modified backbones, and also peptide nucleic acids (PNA) *etc.*

According to a further aspect, the invention provides vectors comprising nucleotide sequences of the invention (*e.g.* expression vectors) and host cells transformed with such vectors.

According to a further aspect, the invention provides compositions comprising protein, antibody, and/or nucleic acid according to the invention. These compositions may be suitable as vaccines, for instance, or as diagnostic reagents, or as immunogenic compositions.

The invention also provides nucleic acid, protein, or antibody according to the invention for use as medicaments (*e.g.* as vaccines or as immunogenic compositions) or as diagnostic reagents. It also provides the use of nucleic acid, protein, or antibody according to the invention in the manufacture of: (i) a medicament for treating or preventing infection due to Neisserial bacteria; (ii) a diagnostic reagent for detecting the presence of Neisserial bacteria or of antibodies raised against Neisserial bacteria; and/or (iii) a reagent which can raise antibodies against Neisserial bacteria. Said Neisserial bacteria may be any species or strain (such as *N.gonorrhoeae*) but are preferably *N.meningitidis*, especially strain A or strain B.

The invention also provides a method of treating a patient, comprising administering to the patient a therapeutically effective amount of nucleic acid, protein, and/or antibody according to the invention.

According to further aspects, the invention provides various processes.

A process for producing proteins of the invention is provided, comprising the step of culturing a host cell according to the invention under conditions which induce protein expression.

A process for producing protein or nucleic acid of the invention is provided, wherein the protein or nucleic acid is synthesised in part or in whole using chemical means.

A process for detecting polynucleotides of the invention is provided, comprising the steps of: (a) contacting a nucleic probe according to the invention with a biological sample under hybridizing conditions to form duplexes; and (b) detecting said duplexes.

A process for detecting proteins of the invention is provided, comprising the steps of: (a) contacting an antibody according to the invention with a biological sample under conditions suitable for the formation of an antibody-antigen complexes; and (b) detecting said complexes.

A summary of standard techniques and procedures which may be employed in order to perform the invention (*e.g.* to utilise the disclosed sequences for vaccination or diagnostic purposes) follows. This summary is not a limitation on the invention but, rather, gives examples that may be used, but are not required.

General

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature *e.g.* Sambrook *Molecular Cloning; A Laboratory Manual, Second Edition* (1989); *DNA Cloning, Volumes I and ii* (D.N Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed, 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds. 1984); *Transcription and Translation* (B.D. Hames & S.J. Higgins eds. 1984); *Animal Cell Culture* (R.I. Freshney ed. 1986); *Immobilized Cells and Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide to Molecular Cloning* (1984); the *Methods in Enzymology* series (Academic Press, Inc.), especially volumes 154 & 155; *Gene Transfer Vectors for Mammalian Cells* (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory); Mayer and Walker, eds. (1987), *Immunochemical Methods in Cell and Molecular Biology* (Academic Press, London); Scopes, (1987) *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.), and *Handbook of Experimental Immunology, Volumes I-IV* (D.M. Weir and C. C. Blackwell eds 1986).

Standard abbreviations for nucleotides and amino acids are used in this specification.

All publications, patents, and patent applications cited herein are incorporated in full by reference.

Definitions

A composition containing X is "substantially free of" Y when at least 85% by weight of the total X+Y in the composition is X. Preferably, X comprises at least about 90% by weight of the total of X+Y in the composition, more preferably at least about 95% or even 99% by weight.

The term "comprising" means "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional to X, such as X+Y.

The term "antigenic determinant" includes B-cell epitopes and T-cell epitopes.

The term "heterologous" refers to two biological components that are not found together in nature. The components may be host cells, genes, or regulatory regions, such as promoters. Although the heterologous components are not found together in nature, they can function together, as when a promoter heterologous to a gene is operably linked to the gene. Another example is where a meningococcal sequence is heterologous to a mouse host cell. A further examples would be two epitopes from the same or different proteins which have been assembled in a single protein in an arrangement not found in nature.

An "origin of replication" is a polynucleotide sequence that initiates and regulates replication of polynucleotides, such as an expression vector. The origin of replication behaves as an autonomous unit of polynucleotide replication within a cell, capable of replication under its own control. An origin of replication may be needed for a vector to replicate in a particular host cell. With certain origins of replication, an expression vector can be reproduced at a high copy number in the presence of the appropriate proteins within the cell. Examples of origins are the autonomously replicating sequences, which are effective in yeast; and the viral T-antigen, effective in COS-7 cells.

Expression systems

The meningococcal nucleotide sequences can be expressed in a variety of different expression systems; for example those used with mammalian cells, baculoviruses, plants, bacteria, and yeast.

i. Mammalian Systems

Mammalian expression systems are known in the art. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence (*e.g.* structural gene) into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, usually located 25-30 base pairs (bp) upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element, usually located within 100 to 200 bp upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation [Sambrook et al. (1989) "Expression of Cloned Genes in Mammalian Cells." In *Molecular Cloning: A Laboratory Manual, 2nd ed.]*.

Mammalian viral genes are often highly expressed and have a broad host range; therefore sequences encoding mammalian viral genes provide particularly useful promoter sequences. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter (Ad MLP), and herpes simplex virus promoter. In addition, sequences derived from non-viral genes, such as the murine metallotheionein gene, also provide useful promoter sequences. Expression may be either constitutive or regulated (inducible), depending on the promoter can be induced with glucocorticoid in hormone-responsive cells.

The presence of an enhancer element (enhancer), combined with the promoter elements described above, will usually increase expression levels. An enhancer is a regulatory DNA sequence that can stimulate transcription up to 1000-fold when linked to homologous or heterologous promoters, with synthesis beginning at the normal RNA start site. Enhancers are also active when they are placed upstream or downstream from the transcription initiation site, in either normal or flipped orientation, or at a distance of more than 1000 nucleotides from the promoter [Maniatis et al. (1987) *Science 236:*1237; Alberts et al. (1989) *Molecular Biology of the Cell*, 2nd ed.]. Enhancer elements derived from viruses may be particularly useful, because they usually have a broader host range. Examples include the SV40 early gene enhancer [Dijkema et al (1985) *EMBO J. 4*:761] and the enhancer/promoters derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus [Gorman et al. (1982b) *Proc. Natl. Acad. Sci. 79*:6777] and from human cytomegalovirus [Boshart et al. (1985) *Cell 41*:521]. Additionally, some enhancers are regulatable and become active only in the presence of an inducer, such as a hormone or metal ion [Sassone-Corsi and Borelli (1986) *Trends Genet. 2*:215; Maniatis et al. (1987) *Science* 236:1237].

A DNA molecule may be expressed intracellularly in mammalian cells. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide.

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either *in vivo* or *in vitro*. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The adenovirus triparite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

Usually, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-transcriptional cleavage and polyadenylation [Birnstiel et al. (1985) *Cell 41*:349; Proudfoot and Whitelaw

(1988) "Termination and 3' end processing of eukaryotic RNA. In *Transcription and splicing* (ed. B.D. Hames and D.M. Glover); Proudfoot (1989) *Trends Biochem. Sci. 14*:105]. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminater/polyadenylation signals include those derived from SV40 [Sambrook et al (1989) "Expression of cloned genes in cultured mammalian cells." In *Molecular Cloning: A Laboratory Manual*].

Usually, the above described components, comprising a promoter, polyadenylation signal, and transcription termination sequence are put together into expression constructs. Enhancers, introns with functional splice donor and acceptor sites, and leader sequences may also be included in an expression construct, if desired. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (*e.g.* plasmids) capable of stable maintenance in a host, such as mammalian cells or bacteria. Mammalian replication systems include those derived from animal viruses, which require trans-acting factors to replicate. For example, plasmids containing the replication systems of papovaviruses, such as SV40 [Gluzman (1981) *Cell 23*:175] or polyomavirus, replicate to extremely high copy number in the presence of the appropriate viral T antigen. Additional examples of mammalian replicons include those derived from bovine papillomavirus and Epstein-Barr virus. Additionally, the replicon may have two replicaton systems, thus allowing it to be maintained, for example, in mammalian cells for expression and in a prokaryotic host for cloning and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2 [Kaufman et al. (1989) *Mol. Cell. Biol. 9*:946] and pHEBO [Shimizu et al. (1986) *Mol. Cell. Biol. 6*:1074].

The transformation procedure used depends upon the host to be transformed. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (*e.g.* Hep G2), and a number of other cell lines.

ii. Baculovirus Systems

The polynucleotide encoding the protein can also be inserted into a suitable insect expression vector, and is operably linked to the control elements within that vector. Vector construction employs techniques which are known in the art. Generally, the components of the expression system include a transfer vector, usually a bacterial plasmid, which contains both a fragment of the baculovirus genome, and a convenient restriction site for insertion of the heterologous gene or genes to be expressed; a wild type baculovirus with a sequence homologous to the baculovirus-specific fragment in the transfer vector (this allows for the homologous

recombination of the heterologous gene in to the baculovirus genome); and appropriate insect host cells and growth media.

After inserting the DNA sequence encoding the protein into the transfer vector, the vector and the wild type viral genome are transfected into an insect host cell where the vector and viral genome are allowed to recombine. The packaged recombinant virus is expressed and recombinant plaques are identified and purified. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego CA ("MaxBac" kit). These techniques are generally known to those skilled in the art and fully described in Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987) (hereinafter "Summers and Smith").

Prior to inserting the DNA sequence encoding the protein into the baculovirus genome, the above described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are usually assembled into an intermediate transplacement construct (transfer vector). This construct may contain a single gene and operably linked regulatory elements; multiple genes, each with its owned set of operably linked regulatory elements; or multiple genes, regulated by the same set of regulatory elements. Intermediate transplacement constructs are often maintained in a replicon, such as an extrachromosomal element (*e.g.* plasmids) capable of stable maintenance in a host, such as a bacterium. The replicon will have a replication system, thus allowing it to be maintained in a suitable host for cloning and amplification.

Currently, the most commonly used transfer vector for introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, have also been designed. These include, for example, pVL985 (which alters the polyhedrin start codon from ATG to ATT, and which introduces a BamHI cloning site 32 basepairs downstream from the ATT; see Luckow and Summers, *Virology* (1989) *17*:31.

The plasmid usually also contains the polyhedrin polyadenylation signal (Miller et al. (1988) *Ann. Rev. Microbiol.*, *42*:177) and a prokaryotic ampicillin-resistance (*amp*) gene and origin of replication for selection and propagation in *E. coli*.

Baculovirus transfer vectors usually contain a baculovirus promoter. A baculovirus promoter is any DNA sequence capable of binding a baculovirus RNA polymerase and initiating the downstream (5' to 3') transcription of a coding sequence (*e.g.* structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A baculovirus transfer vector may also have a second domain called an enhancer, which, if present, is usually distal to the structural gene. Expression may be either regulated or constitutive.

Structural genes, abundantly transcribed at late times in a viral infection cycle, provide particularly useful promoter sequences. Examples include sequences derived from the gene encoding the viral polyhedron

protein, Friesen et al., (1986) "The Regulation of Baculovirus Gene Expression," in: *The Molecular Biology of Baculoviruses* (ed. Walter Doerfler); EPO Publ. Nos. 127 839 and 155 476; and the gene encoding the p10 protein, Vlak et al., (1988), *J. Gen. Virol. 69*:765.

DNA encoding suitable signal sequences can be derived from genes for secreted insect or baculovirus proteins, such as the baculovirus polyhedrin gene (Carbonell et al. (1988) *Gene*, *73*:409). Alternatively, since the signals for mammalian cell posttranslational modifications (such as signal peptide cleavage, proteolytic cleavage, and phosphorylation) appear to be recognized by insect cells, and the signals required for secretion and nuclear accumulation also appear to be conserved between the invertebrate cells and vertebrate cells, leaders of non-insect origin, such as those derived from genes encoding human α-interferon, Maeda et al., (1985), *Nature 315*:592; human gastrin-releasing peptide, Lebacq-Verheyden et al., (1988), *Molec. Cell. Biol. 8*:3129; human IL-2, Smith et al., (1985) *Proc. Nat'l Acad. Sci. USA*, *82*:8404; mouse IL-3, (Miyajima et al., (1987) *Gene 58*:273; and human glucocerebrosidase, Martin et al. (1988) *DNA*, *7*:99, can also be used to provide for secretion in insects.

A recombinant polypeptide or polyprotein may be expressed intracellularly or, if it is expressed with the proper regulatory sequences, it can be secreted. Good intracellular expression of nonfused foreign proteins usually requires heterologous genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal. If desired, methionine at the N-terminus may be cleaved from the mature protein by *in vitro* incubation with cyanogen bromide.

Alternatively, recombinant polyproteins or proteins which are not naturally secreted can be secreted from the insect cell by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in insects. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the translocation of the protein into the endoplasmic reticulum.

After insertion of the DNA sequence and/or the gene encoding the expression product precursor of the protein, an insect cell host is co-transformed with the heterologous DNA of the transfer vector and the genomic DNA of wild type baculovirus -- usually by co-transfection. The promoter and transcription termination sequence of the construct will usually comprise a 2-5kb section of the baculovirus genome. Methods for introducing heterologous DNA into the desired site in the baculovirus virus are known in the art. (See Summers and Smith *supra*; Ju et al. (1987); Smith et al., *Mol. Cell. Biol.* (1983) *3*:2156; and Luckow and Summers (1989)). For example, the insertion can be into a gene such as the polyhedrin gene, by homologous double crossover recombination; insertion can also be into a restriction enzyme site engineered into the desired baculovirus gene. Miller et al., (1989), *Bioessays 4*:91.The DNA sequence, when cloned in place of the polyhedrin gene in the expression vector, is flanked both 5' and 3' by polyhedrin-specific sequences and is positioned downstream of the polyhedrin promoter.

The newly formed baculovirus expression vector is subsequently packaged into an infectious recombinant baculovirus. Homologous recombination occurs at low frequency (between about 1% and about 5%); thus, the majority of the virus produced after cotransfection is still wild-type virus. Therefore, a method is necessary to identify recombinant viruses. An advantage of the expression system is a visual screen allowing recombinant viruses to be distinguished. The polyhedrin protein, which is produced by the native virus, is produced at very high levels in the nuclei of infected cells at late times after viral infection. Accumulated polyhedrin protein forms occlusion bodies that also contain embedded particles. These occlusion bodies, up to 15 μm in size, are highly refractile, giving them a bright shiny appearance that is readily visualized under the light microscope. Cells infected with recombinant viruses lack occlusion bodies. To distinguish recombinant virus from wild-type virus, the transfection supernatant is plaqued onto a monolayer of insect cells by techniques known to those skilled in the art. Namely, the plaques are screened under the light microscope for the presence (indicative of wild-type virus) or absence (indicative of recombinant virus) of occlusion bodies. "Current Protocols in Microbiology" Vol. 2 (Ausubel et al. eds) at 16.8 (Supp. 10, 1990); Summers and Smith, *supra*; Miller et al. (1989).

Recombinant baculovirus expression vectors have been developed for infection into several insect cells. For example, recombinant baculoviruses have been developed for, *inter alia*: *Aedes aegypti* , *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni* (WO 89/046699; Carbonell et al., (1985) *J. Virol. 56*:153; Wright (1986) *Nature 321*:718; Smith et al., (1983) *Mol. Cell. Biol. 3*:2156; and see generally, Fraser, *et al.* (1989) *In Vitro Cell. Dev. Biol. 25*:225).

Cells and cell culture media are commercially available for both direct and fusion expression of heterologous polypeptides in a baculovirus/expression system; cell culture technology is generally known to those skilled in the art. *See, e.g.* Summers and Smith *supra.*

The modified insect cells may then be grown in an appropriate nutrient medium, which allows for stable maintenance of the plasmid(s) present in the modified insect host. Where the expression product gene is under inducible control, the host may be grown to high density, and expression induced. Alternatively, where expression is constitutive, the product will be continuously expressed into the medium and the nutrient medium must be continuously circulated, while removing the product of interest and augmenting depleted nutrients. The product may be purified by such techniques as chromatography, e.g. HPLC, affinity chromatography, ion exchange chromatography, etc.; electrophoresis; density gradient centrifugation; solvent extraction, or the like. As appropriate, the product may be further purified, as required, so as to remove substantially any insect proteins which are also secreted in the medium or result from lysis of insect cells, so as to provide a product which is at least substantially free of host debris, e.g. proteins, lipids and polysaccharides.

In order to obtain protein expression, recombinant host cells derived from the transformants are incubated under conditions which allow expression of the recombinant protein encoding sequence. These conditions

will vary, dependent upon the host cell selected. However, the conditions are readily ascertainable to those of ordinary skill in the art, based upon what is known in the art.

iii. Plant Systems

There are many plant cell culture and whole plant genetic expression systems known in the art. Exemplary plant cellular genetic expression systems include those described in patents, such as: US 5,693,506; US 5,659,122; and US 5,608,143. Additional examples of genetic expression in plant cell culture has been described by Zenk, *Phytochemistry* 30:3861-3863 (1991). Descriptions of plant protein signal peptides may be found in addition to the references described above in Vaulcombe et al., *Mol. Gen. Genet.* 209:33-40 (1987); Chandler et al., *Plant Molecular Biology* 3:407-418 (1984); Rogers, *J. Biol. Chem.* 260:3731-3738 (1985); Rothstein et al., *Gene* 55:353-356 (1987); Whittier et al., Nucleic Acids Research 15:2515-2535 (1987); Wirsel et al., *Molecular Microbiology* 3:3-14 (1989); Yu et al., *Gene* 122:247-253 (1992). A description of the regulation of plant gene expression by the phytohormone, gibberellic acid and secreted enzymes induced by gibberellic acid can be found in R.L. Jones and J. MacMillin, Gibberellins: in: *Advanced Plant Physiology*,. Malcolm B. Wilkins, ed., 1984 Pitman Publishing Limited, London, pp. 21-52. References that describe other metabolically-regulated genes: Sheen, *Plant Cell*, 2:1027-1038(1990); Maas et al., *EMBO J.* 9:3447-3452 (1990); Benkel and Hickey, *Proc. Natl. Acad. Sci.* 84:1337-1339 (1987)

Typically, using techniques known in the art, a desired polynucleotide sequence is inserted into an expression cassette comprising genetic regulatory elements designed for operation in plants. The expression cassette is inserted into a desired expression vector with companion sequences upstream and downstream from the expression cassette suitable for expression in a plant host. The companion sequences will be of plasmid or viral origin and provide necessary characteristics to the vector to permit the vectors to move DNA from an original cloning host, such as bacteria, to the desired plant host. The basic bacterial/plant vector construct will preferably provide a broad host range prokaryote replication origin; a prokaryote selectable marker; and, for Agrobacterium transformations, T DNA sequences for Agrobacterium-mediated transfer to plant chromosomes. Where the heterologous gene is not readily amenable to detection, the construct will preferably also have a selectable marker gene suitable for determining if a plant cell has been transformed. A general review of suitable markers, for example for the members of the grass family, is found in Wilmink and Dons, 1993, *Plant Mol. Biol. Reptr*, 11(2):165-185.

Sequences suitable for permitting integration of the heterologous sequence into the plant genome are also recommended. These might include transposon sequences and the like for homologous recombination as well as Ti sequences which permit random insertion of a heterologous expression cassette into a plant genome. Suitable prokaryote selectable markers include resistance toward antibiotics such as ampicillin or tetracycline. Other DNA sequences encoding additional functions may also be present in the vector, as is known in the art.

The nucleic acid molecules of the subject invention may be included into an expression cassette for expression of the protein(s) of interest. Usually, there will be only one expression cassette, although two or more are feasible. The recombinant expression cassette will contain in addition to the heterologous protein encoding sequence the following elements, a promoter region, plant 5' untranslated sequences, initiation codon depending upon whether or not the structural gene comes equipped with one, and a transcription and translation termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the cassette allow for easy insertion into a pre-existing vector.

A heterologous coding sequence may be for any protein relating to the present invention. The sequence encoding the protein of interest will encode a signal peptide which allows processing and translocation of the protein, as appropriate, and will usually lack any sequence which might result in the binding of the desired protein of the invention to a membrane. Since, for the most part, the transcriptional initiation region will be for a gene which is expressed and translocated during germination, by employing the signal peptide which provides for translocation, one may also provide for translocation of the protein of interest. In this way, the protein(s) of interest will be translocated from the cells in which they are expressed and may be efficiently harvested. Typically secretion in seeds are across the aleurone or scutellar epithelium layer into the endosperm of the seed. While it is not required that the protein be secreted from the cells in which the protein is produced, this facilitates the isolation and purification of the recombinant protein.

Since the ultimate expression of the desired gene product will be in a eucaryotic cell it is desirable to determine whether any portion of the cloned gene contains sequences which will be processed out as introns by the host's splicosome machinery. If so, site-directed mutagenesis of the "intron" region may be conducted to prevent losing a portion of the genetic message as a false intron code, Reed and Maniatis, *Cell* 41:95-105, 1985.

The vector can be microinjected directly into plant cells by use of micropipettes to mechanically transfer the recombinant DNA. Crossway, *Mol. Gen. Genet*, 202:179-185, 1985. The genetic material may also be transferred into the plant cell by using polyethylene glycol, Krens, et al., *Nature*, 296, 72-74, 1982. Another method of introduction of nucleic acid segments is high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface, Klein, et al., *Nature*, 327, 70-73, 1987 and Knudsen and Muller, 1991, *Planta*, 185:330-336 teaching particle bombardment of barley endosperm to create transgenic barley. Yet another method of introduction would be fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies, Fraley, et al., *Proc. Natl. Acad. Sci. USA*, 79, 1859-1863, 1982.

The vector may also be introduced into the plant cells by electroporation. (Fromm et al., *Proc. Natl Acad. Sci. USA* 82:5824, 1985). In this technique, plant protoplasts are electroporated in the presence of plasmids containing the gene construct. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and form plant callus.

All plants from which protoplasts can be isolated and cultured to give whole regenerated plants can be transformed by the present invention so that whole plants are recovered which contain the transferred gene. It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to all major species of sugarcane, sugar beet, cotton, fruit and other trees, legumes and vegetables. Some suitable plants include, for example, species from the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersion*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Cichorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Hererocallis*, *Nemesia*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browaalia*, *Glycine*, *Lolium*, *Zea*, *Triticum*, *Sorghum*, and *Datura*.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts containing copies of the heterologous gene is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced from the protoplast suspension. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Shoots and roots normally develop simultaneously. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is fully reproducible and repeatable.

In some plant cell culture systems, the desired protein of the invention may be excreted or alternatively, the protein may be extracted from the whole plant. Where the desired protein of the invention is secreted into the medium, it may be collected. Alternatively, the embryos and embryoless-half seeds or other plant tissue may be mechanically disrupted to release any secreted protein between cells and tissues. The mixture may be suspended in a buffer solution to retrieve soluble proteins. Conventional protein isolation and purification methods will be then used to purify the recombinant protein. Parameters of time, temperature pH, oxygen, and volumes will be adjusted through routine methods to optimize expression and recovery of heterologous protein.

iv. Bacterial Systems

Bacterial expression techniques are known in the art. A bacterial promoter is any DNA sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of a coding sequence (*e.g.* structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A bacterial promoter may also have a second domain called an operator, that may overlap an adjacent RNA polymerase binding site at which RNA synthesis begins. The operator permits negative regulated (inducible) transcription, as a gene repressor protein may bind the operator and thereby inhibit transcription of a specific gene. Constitutive expression

may occur in the absence of negative regulatory elements, such as the operator. In addition, positive regulation may be achieved by a gene activator protein binding sequence, which, if present is usually proximal (5') to the RNA polymerase binding sequence. An example of a gene activator protein is the catabolite activator protein (CAP), which helps initiate transcription of the lac operon in Escherichia coli (E. coli) [Raibaud *et al.* (1984) *Annu. Rev. Genet. 18*:173]. Regulated expression may therefore be either positive or negative, thereby either enhancing or reducing transcription.

Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) [Chang *et al.* (1977) *Nature 198*:1056], and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*) [Goeddel *et al.* (1980) *Nuc. Acids Res. 8*:4057; Yelverton *et al.* (1981) *Nucl. Acids Res. 9*:731; US patent 4,738,921; EP-A-0036776 and EP-A-0121775]. The g-laotamase (*bla*) promoter system [Weissmann (1981) "The cloning of interferon and other mistakes." In *Interferon 3* (ed. I. Gresser)], bacteriophage lambda PL [Shimatake *et al.* (1981) *Nature 292*:128] and T5 [US patent 4,689,406] promoter systems also provide useful promoter sequences.

In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one bacterial or bacteriophage promoter may be joined with the operon sequences of another bacterial or bacteriophage promoter, creating a synthetic hybrid promoter [US patent 4,551,433]. For example, the *tac* promoter is a hybrid *trp-lac* promoter comprised of both *trp* promoter and *lac* operon sequences that is regulated by the *lac* repressor [Amann *et al.* (1983) *Gene 25*:167; de Boer *et al.* (1983) *Proc. Natl. Acad. Sci. 80*:21]. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. A naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of some genes in prokaryotes. The bacteriophage T7 RNA polymerase/promoter system is an example of a coupled promoter system [Studier *et al.* (1986) *J. Mol. Biol. 189*:113; Tabor *et al.* (1985) *Proc Natl. Acad. Sci. 82*:1074]. In addition, a hybrid promoter can also be comprised of a bacteriophage promoter and an *E. coli* operator region (EP-A-0267851).

In addition to a functioning promoter sequence, an efficient ribosome binding site is also useful for the expression of foreign genes in prokaryotes. In *E. coli*, the ribosome binding site is called the Shine-Dalgarno (SD) sequence and includes an initiation codon (ATG) and a sequence 3-9 nucleotides in length located 3-11 nucleotides upstream of the initiation codon [Shine *et al.* (1975) *Nature 254*:34]. The SD sequence is thought to promote binding of mRNA to the ribosome by the pairing of bases between the SD sequence and the 3' and of *E. coli* 16S rRNA [Steitz *et al.* (1979) "Genetic signals and nucleotide sequences in messenger RNA." In *Biological Regulation and Development: Gene Expression* (ed. R.F. Goldberger)]. To express eukaryotic genes and prokaryotic genes with weak ribosome-binding site [Sambrook *et al.* (1989) "Expression of cloned genes in Escherichia coli." In *Molecular Cloning: A Laboratory Manual*].

A DNA molecule may be expressed intracellularly. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide or by either *in vivo* on *in vitro* incubation with a bacterial methionine N-terminal peptidase (EPO-A-0 219 237).

Fusion proteins provide an alternative to direct expression. Usually, a DNA sequence encoding the N-terminal portion of an endogenous bacterial protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the bacteriophage lambda cell gene can be linked at the 5' terminus of a foreign gene and expressed in bacteria. The resulting fusion protein preferably retains a site for a processing enzyme (factor Xa) to cleave the bacteriophage protein from the foreign gene [Nagai *et al.* (1984) *Nature 309*:810]. Fusion proteins can also be made with sequences from the *lacZ* [Jia *et al.* (1987) *Gene 60*:197], *trpE* [Allen et al. (1987) J. Biotechnol. 5:93; Makoff *et al.* (1989) *J. Gen. Microbiol. 135*:11], and *Chey* [EP-A-0 324 647] genes. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a processing enzyme (*e.g.* ubiquitin specific processing-protease) to cleave the ubiquitin from the foreign protein. Through this method, native foreign protein can be isolated [Miller *et al.* (1989) *Bio/Technology 7*:698].

Alternatively, foreign proteins can also be secreted from the cell by creating chimeric DNA molecules that encode a fusion protein comprised of a signal peptide sequence fragment that provides for secretion of the foreign protein in bacteria [US patent 4,336,336]. The signal sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria). Preferably there are processing sites, which can be cleaved either *in vivo* or *in vitro* encoded between the signal peptide fragment and the foreign gene.

DNA encoding suitable signal sequences can be derived from genes for secreted bacterial proteins, such as the *E. coli* outer membrane protein gene (*ompA*) [Masui *et al.* (1983), in: *Experimental Manipulation of Gene Expression*; Ghrayeb *et al.* (1984) *EMBO J. 3*:2437] and the *E. coli* alkaline phosphatase signal sequence (*phoA*) [Oka *et al.* (1985) *Proc. Natl. Acad. Sci. 82*:7212]. As an additional example, the signal sequence of the alpha-amylase gene from various Bacillus strains can be used to secrete heterologous proteins from *B. subtilis* [Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA 79*:5582; EP-A-0 244 042].

Usually, transcription termination sequences recognized by bacteria are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA.

Transcription termination sequences frequently include DNA sequences of about 50 nucleotides capable of forming stem loop structures that aid in terminating transcription. Examples include transcription termination sequences derived from genes with strong promoters, such as the *trp* gene in *E. coli* as well as other biosynthetic genes.

Usually, the above described components, comprising a promoter, signal sequence (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (*e.g.* plasmids) capable of stable maintenance in a host, such as bacteria. The replicon will have a replication system, thus allowing it to be maintained in a prokaryotic host either for expression or for cloning and amplification. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably contain at least about 10, and more preferably at least about 20 plasmids. Either a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host.

Alternatively, the expression constructs can be integrated into the bacterial genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to the bacterial chromosome that allows the vector to integrate. Integrations appear to result from recombinations between homologous DNA in the vector and the bacterial chromosome. For example, integrating vectors constructed with DNA from various Bacillus strains integrate into the Bacillus chromosome (EP-A- 0 127 328). Integrating vectors may also be comprised of bacteriophage or transposon sequences.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of bacterial strains that have been transformed. Selectable markers can be expressed in the bacterial host and may include genes which render bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin (neomycin), and tetracycline [Davies *et al.* (1978) *Annu. Rev. Microbiol. 32*:469]. Selectable markers may also include biosynthetic genes, such as those in the histidine, tryptophan, and leucine biosynthetic pathways.

Alternatively, some of the above described components can be put together in transformation vectors. Transformation vectors are usually comprised of a selectable market that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extra-chromosomal replicons or integrating vectors, have been developed for transformation into many bacteria. For example, expression vectors have been developed for, *inter alia*, the following bacteria: Bacillus subtilis [Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA 79*:5582; EP-A-0 036 259 and EP-A-0 063 953; WO 84/04541], Escherichia coli [Shimatake *et al.* (1981) *Nature 292*:128; Amann *et al.* (1985) *Gene 40*:183; Studier *et al.* (1986) *J. Mol. Biol. 189*:113; EP-A-0 036 776,EP-A-0 136 829 and EP-A-0 136 907], Streptococcus cremoris [Powell *et al.* (1988) *Appl. Environ. Microbiol.*

54:655]; Streptococcus lividans [Powell *et al.* (1988) *Appl. Environ. Microbiol. 54*:655], Streptomyces lividans [US patent 4,745,056].

Methods of introducing exogenous DNA into bacterial hosts are well-known in the art, and usually include either the transformation of bacteria treated with $CaCl_2$ or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation. Transformation procedures usually vary with the bacterial species to be transformed. See *e.g.* [Masson *et al.* (1989) *FEMS Microbiol. Lett. 60*:273; Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA 79*:5582; EP-A-0 036 259 and EP-A-0 063 953; WO 84/04541, Bacillus], [Miller *et al.* (1988) *Proc. Natl. Acad. Sci. 85*:856; Wang *et al.* (1990) *J. Bacteriol. 172*:949, Campylobacter], [Cohen *et al.* (1973) *Proc. Natl. Acad. Sci. 69*:2110; Dower *et al.* (1988) *Nucleic Acids Res. 16*:6127; Kushner (1978) "An improved method for transformation of Escherichia coli with ColE1-derived plasmids. In *Genetic Engineering: Proceedings of the International Symposium on Genetic Engineering* (eds. H.W. Boyer and S. Nicosia); Mandel *et al.* (1970) *J. Mol. Biol. 53*:159; Taketo (1988) *Biochim. Biophys. Acta 949*:318; Escherichia], [Chassy *et al.* (1987) *FEMS Microbiol. Lett. 44*:173 Lactobacillus]; [Fiedler *et al.* (1988) *Anal. Biochem 170*:38, Pseudomonas]; [Augustin *et al.* (1990) *FEMS Microbiol. Lett. 66*:203, Staphylococcus], [Barany *et al.* (1980) *J. Bacteriol. 144*:698; Harlander (1987) "Transformation of Streptococcus lactis by electroporation, in: *Streptococcal Genetics* (ed. J. Ferretti and R. Curtiss III); Perry *et al.* (1981) *Infect. Immun. 32*:1295; Powell *et al.* (1988) *Appl. Environ. Microbiol. 54*:655; Somkuti *et al.* (1987) *Proc. 4th Evr. Cong. Biotechnology 1*:412, Streptococcus].

v. Yeast Expression

Yeast expression systems are also known to one of ordinary skill in the art. A yeast promoter is any DNA sequence capable of binding yeast RNA polymerase and initiating the downstream (3') transcription of a coding sequence (*e.g.* structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site (the "TATA Box") and a transcription initiation site. A yeast promoter may also have a second domain called an upstream activator sequence (UAS), which, if present, is usually distal to the structural gene. The UAS permits regulated (inducible) expression. Constitutive expression occurs in the absence of a UAS. Regulated expression may be either positive or negative, thereby either enhancing or reducing transcription.

Yeast is a fermenting organism with an active metabolic pathway, therefore sequences encoding enzymes in the metabolic pathway provide particularly useful promoter sequences. Examples include alcohol dehydrogenase (ADH) (EP-A-0284044), glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), enolase, glucokinase, hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK) (EP-A-0329203). The yeast *PHO5* gene, encoding acid phosphatase, also provides useful promoter sequences [Myanohara *et al.* (1983) *Proc. Natl. Acad. Sci. USA 80*:1].

In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For example, UAS sequences of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region (US Patent Nos. 4,876,197 and 4,880,734). Other examples of hybrid promoters include promoters which consist of the regulatory sequences of either the *ADH2*, *GAL4*, *GAL10*, OR *PHO5* genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as GAP or PyK (EP-A-0 164 556). Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription. Examples of such promoters include, *inter alia*, [Cohen *et al.* (1980) *Proc. Natl. Acad. Sci. USA 77*:1078; Henikoff *et al.* (1981) *Nature 283*:835; Hollenberg *et al.* (1981) *Curr. Topics Microbiol. Immunol. 96*:119; Hollenberg *et al.* (1979) "The Expression of Bacterial Antibiotic Resistance Genes in the Yeast Saccharomyces cerevisiae," in: *Plasmids of Medical, Environmental and Commercial Importance* (eds. K.N. Timmis and A. Puhler); Mercerau-Puigalon *et al.* (1980) *Gene 11*:163; Panthier *et al.* (1980) *Curr. Genet. 2*:109;].

A DNA molecule may be expressed intracellularly in yeast. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide.

Fusion proteins provide an alternative for yeast expression systems, as well as in mammalian, baculovirus, and bacterial expression systems. Usually, a DNA sequence encoding the N-terminal portion of an endogenous yeast protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the yeast or human superoxide dismutase (SOD) gene, can be linked at the 5' terminus of a foreign gene and expressed in yeast. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. See *e.g.* EP-A-0 196 056. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a processing enzyme (*e.g.* ubiquitin-specific processing protease) to cleave the ubiquitin from the foreign protein. Through this method, therefore, native foreign protein can be isolated (*e.g.* WO88/024066).

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provide for secretion in yeast of the foreign protein. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either *in vivo* or *in vitro*. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell.

DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast invertase gene (EP-A-0 012 873; JPO. 62,096,086) and the A-factor gene (US patent 4,588,684). Alternatively, leaders of non-yeast origin, such as an interferon leader, exist that also provide for secretion in yeast (EP-A-0 060 057).

A preferred class of secretion leaders are those that employ a fragment of the yeast alpha-factor gene, which contains both a "pre" signal sequence, and a "pro" region. The types of alpha-factor fragments that can be employed include the full-length pre-pro alpha factor leader (about 83 amino acid residues) as well as truncated alpha-factor leaders (usually about 25 to about 50 amino acid residues) (US Patents 4,546,083 and 4,870,008; EP-A-0 324 274). Additional leaders employing an alpha-factor leader fragment that provides for secretion include hybrid alpha-factor leaders made with a presequence of a first yeast, but a pro-region from a second yeast alphafactor. (*e.g.* see WO 89/02463.)

Usually, transcription termination sequences recognized by yeast are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator sequence and other yeast-recognized termination sequences, such as those coding for glycolytic enzymes.

Usually, the above described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (*e.g.* plasmids) capable of stable maintenance in a host, such as yeast or bacteria. The replicon may have two replication systems, thus allowing it to be maintained, for example, in yeast for expression and in a prokaryotic host for cloning and amplification. Examples of such yeast-bacteria shuttle vectors include YEp24 [Botstein *et al.* (1979) *Gene 8*:17-24], pCl/1 [Brake *et al.* (1984) *Proc. Natl. Acad. Sci USA 81*:4642-4646], and YRp17 [Stinchcomb *et al.* (1982) *J. Mol. Biol. 158*:157]. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably have at least about 10, and more preferably at least about 20. Enter a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host. See *e.g.* Brake *et al.*, *supra.*

Alternatively, the expression constructs can be integrated into the yeast genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to a yeast chromosome that allows the vector to integrate, and preferably contain two homologous sequences flanking the expression construct. Integrations appear to result from recombinations between homologous DNA in the vector and the yeast chromosome [Orr-Weaver *et al.* (1983) *Methods in Enzymol. 101*:228-245]. An integrating vector may be directed to a specific locus in yeast by selecting the appropriate homologous sequence for inclusion in the vector. See Orr-Weaver *et al.*, *supra.* One or more expression construct may integrate, possibly affecting

levels of recombinant protein produced [Rine *et al.* (1983) *Proc. Natl. Acad. Sci. USA 80*:6750]. The chromosomal sequences included in the vector can occur either as a single segment in the vector, which results in the integration of the entire vector, or two segments homologous to adjacent segments in the chromosome and flanking the expression construct in the vector, which can result in the stable integration of only the expression construct.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of yeast strains that have been transformed. Selectable markers may include biosynthetic genes that can be expressed in the yeast host, such as *ADE2*, *HIS4*, *LEU2*, *TRP1*, and *ALG7*, and the G418 resistance gene, which confer resistance in yeast cells to tunicamycin and G418, respectively. In addition, a suitable selectable marker may also provide yeast with the ability to grow in the presence of toxic compounds, such as metal. For example, the presence of *CUP1* allows yeast to grow in the presence of copper ions [Butt *et al.* (1987) *Microbiol, Rev. 51*:351].

Alternatively, some of the above described components can be put together into transformation vectors. Transformation vectors are usually comprised of a selectable marker that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example, expression vectors have been developed for, *inter alia*, the following yeasts:Candida albicans [Kurtz, *et al.* (1986) *Mol. Cell. Biol. 6*:142], Candida maltosa [Kunze, *et al.* (1985) *J. Basic Microbiol. 25*:141]. Hansenula polymorpha [Gleeson, *et al.* (1986) *J. Gen. Microbiol. 132*:3459; Roggenkamp *et al.* (1986) *Mol. Gen. Genet. 202*:302], Kluyveromyces fragilis [Das, *et al.* (1984) *J. Bacteriol. 158*:1165], Kluyveromyces lactis [De Louvencourt *et al.* (1983) *J. Bacteriol. 154*:737; Van den Berg *et al.* (1990) *Bio/Technology 8*:135], Pichia guillerimondii [Kunze *et al.* (1985) *J. Basic Microbiol. 25*:141], Pichia pastoris [Cregg, *et al.* (1985) *Mol. Cell. Biol. 5*:3376; US Patent Nos. 4,837,148 and 4,929,555], Saccharomyces cerevisiae [Hinnen *et al.* (1978) *Proc. Natl. Acad. Sci. USA 75*:1929; Ito *et al.* (1983) *J. Bacteriol. 153*:163], Schizosaccharomyces pombe [Beach and Nurse (1981) *Nature 300*:706], and Yarrowia lipolytica [Davidow, *et al.* (1985) *Curr. Genet. 10*:380471 Gaillardin, *et al.* (1985) *Curr. Genet. 10*:49].

Methods of introducing exogenous DNA into yeast hosts are well-known in the art, and usually include either the transformation of spheroplasts or of intact yeast cells treated with alkali cations. Transformation procedures usually vary with the yeast species to be transformed. See *e.g.* [Kurtz *et al.* (1986) *Mol. Cell. Biol. 6*:142; Kunze *et al.* (1985) *J. Basic Microbiol. 25*:141; Candida]; [Gleeson *et al.* (1986) *J. Gen. Microbiol. 132*:3459; Roggenkamp *et al.* (1986) *Mol. Gen. Genet. 202*:302; Hansenula]; [Das *et al.* (1984) *J. Bacteriol. 158*:1165; De Louvencourt *et al.* (1983) *J. Bacteriol. 154*:1165; Van den Berg *et al.* (1990) *Bio/Technology 8*:135; Kluyveromyces]; [Cregg *et al.* (1985) *Mol. Cell. Biol. 5*:3376; Kunze *et al.* (1985) *J. Basic Microbiol. 25*:141; US Patent Nos. 4,837,148 and 4,929,555; Pichia]; [Hinnen *et al.* (1978) *Proc. Natl.*

Acad. Sci. USA 75;1929; Ito *et al.* (1983) *J. Bacteriol. 153*:163 Saccharomyces]; [Beach and Nurse (1981) *Nature 300*:706; Schizosaccharomyces]; [Davidow *et al.* (1985) *Curr. Genet. 10*:39; Gaillardin *et al.* (1985) *Curr. Genet. 10*:49; Yarrowia].

Antibodies

As used herein, the term "antibody" refers to a polypeptide or group of polypeptides composed of at least one antibody combining site. An "antibody combining site" is the three-dimensional binding space with an internal surface shape and charge distribution complementary to the features of an epitope of an antigen, which allows a binding of the antibody with the antigen. "Antibody" includes, for example, vertebrate antibodies, hybrid antibodies, chimeric antibodies, humanised antibodies, altered antibodies, univalent antibodies, Fab proteins, and single domain antibodies.

Antibodies against the proteins of the invention are useful for affinity chromatography, immunoassays, and distinguishing/identifying meningococcal proteins.

Antibodies to the proteins of the invention, both polyclonal and monoclonal, may be prepared by conventional methods. In general, the protein is first used to immunize a suitable animal, preferably a mouse, rat, rabbit or goat. Rabbits and goats are preferred for the preparation of polyclonal sera due to the volume of serum obtainable, and the availability of labeled anti-rabbit and anti-goat antibodies. Immunization is generally performed by mixing or emulsifying the protein in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 μg/injection is typically sufficient. Immunization is generally boosted 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by in vitro immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vivo* immunization. Polyclonal antisera is obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation (*e.g.* 1,000*g* for 10 minutes). About 20-50 ml per bleed may be obtained from rabbits.

Monoclonal antibodies are prepared using the standard method of Kohler & Milstein [*Nature* (1975) 256:495-96], or a modification thereof. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) is removed and dissociated into single cells. If desired, the spleen cells may be screened (after removal of nonspecifically adherent cells) by applying a cell suspension to a plate or well coated with the protein antigen. B-cells expressing membrane-bound immunoglobulin specific for the antigen bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (*e.g.* hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not

bind to unrelated antigens). The selected MAb-secreting hybridomas are then cultured either *in vitro* (*e.g.* in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice).

If desired, the antibodies (whether polyclonal or monoclonal) may be labeled using conventional techniques. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ^{32}P and ^{125}I), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase is usually detected by its ability to convert 3,3',5,5'-tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. "Specific binding partner" refers to a protein capable of binding a ligand molecule with high specificity, as for example in the case of an antigen and a monoclonal antibody specific therefor. Other specific binding partners include biotin and avidin or streptavidin, IgG and protein A, and the numerous receptor-ligand couples known in the art. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may serve in several different modes. For example, ^{125}I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as enzyme or as antigen for a MAb. Further, one may combine various labels for desired effect. For example, MAbs and avidin also require labels in the practice of this invention: thus, one might label a MAb with biotin, and detect its presence with avidin labeled with ^{125}I, or with an anti-biotin MAb labeled with HRP. Other permutations and possibilities will be readily apparent to those of ordinary skill in the art, and are considered as equivalents within the scope of the invention.

Pharmaceutical Compositions

Pharmaceutical compositions can comprise either polypeptides, antibodies, or nucleic acid of the invention. The pharmaceutical compositions will comprise a therapeutically effective amount of either polypeptides, antibodies, or polynucleotides of the claimed invention.

The term "therapeutically effective amount" as used herein refers to an amount of a therapeutic agent to treat, ameliorate, or prevent a desired disease or condition, or to exhibit a detectable therapeutic or preventative effect. The effect can be detected by, for example, chemical markers or antigen levels. Therapeutic effects also include reduction in physical symptoms, such as decreased body temperature. The precise effective amount for a subject will depend upon the subject's size and health, the nature and extent of the condition, and the therapeutics or combination of therapeutics selected for administration. Thus, it is not useful to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by routine experimentation and is within the judgement of the clinician.

For purposes of the present invention, an effective dose will be from about 0.01 mg/ kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of the DNA constructs in the individual to which it is administered.

A pharmaceutical composition can also contain a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent, such as antibodies or a polypeptide, genes, and other therapeutic agents. The term refers to any pharmaceutical

carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Suitable carriers may be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art.

Pharmaceutically acceptable salts can be used therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

Pharmaceutically acceptable carriers in therapeutic compositions may contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. Liposomes are included within the definition of a pharmaceutically acceptable carrier.

Delivery Methods

Once formulated, the compositions of the invention can be administered directly to the subject. The subjects to be treated can be animals; in particular, human subjects can be treated.

Direct delivery of the compositions will generally be accomplished by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly or delivered to the interstitial space of a tissue. The compositions can also be administered into a lesion. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal or transcutaneous applications (*e.g.* see WO98/20734), needles, and gene guns or hyposprays. Dosage treatment may be a single dose schedule or a multiple dose schedule.

Vaccines

Vaccines according to the invention may either be prophylactic (*i.e.* to prevent infection) or therapeutic (*i.e.* to treat disease after infection).

Such vaccines comprise immunising antigen(s), immunogen(s), polypeptide(s), protein(s) or nucleic acid, usually in combination with "pharmaceutically acceptable carriers," which include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore,

the antigen or immunogen may be conjugated to a bacterial toxoid, such as a toxoid from diphtheria, tetanus, cholera, *H. pylori*, *etc.* pathogens.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59™ (WO 90/14837; Chapter 10 in *Vaccine design: the subunit and adjuvant approach*, eds. Powell & Newman, Plenum Press 1995), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (5) cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, *etc.*), interferons (*e.g.* gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc; and (6) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Alum and MF59™ are preferred.

As mentioned above, muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), *etc.*

The immunogenic compositions (*e.g.* the immunising antigen/immunogen/polypeptide/protein/ nucleic acid, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect, as discussed above under pharmaceutically acceptable carriers.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic or immunogenic polypeptides, as well as any other of the above-mentioned components, as needed. By

"immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (*e.g.* nonhuman primate, primate, *etc.*), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The immunogenic compositions are conventionally administered parenterally, *e.g.* by injection, either subcutaneously, intramuscularly, or transdermally/transcutaneously (*e.g.* WO98/20734). Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories, and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

As an alternative to protein-based vaccines, DNA vaccination may be employed [*e.g.* Robinson & Torres (1997) *Seminars in Immunology* 9:271-283; Donnelly *et al.* (1997) *Annu Rev Immunol* 15:617-648; see later herein].

Gene Delivery Vehicles

Gene therapy vehicles for delivery of constructs including a coding sequence of a therapeutic of the invention, to be delivered to the mammal for expression in the mammal, can be administered either locally or systemically. These constructs can utilize viral or non-viral vector approaches in *in vivo* or *ex vivo* modality. Expression of such coding sequence can be induced using endogenous mammalian or heterologous promoters. Expression of the coding sequence in vivo can be either constitutive or regulated.

The invention includes gene delivery vehicles capable of expressing the contemplated nucleic acid sequences. The gene delivery vehicle is preferably a viral vector and, more preferably, a retroviral, adenoviral, adeno-associated viral (AAV), herpes viral, or alphavirus vector. The viral vector can also be an astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, or togavirus viral vector. See generally, Jolly (1994) *Cancer Gene Therapy* 1:51-64; Kimura (1994) *Human Gene Therapy* 5:845-852; Connelly (1995) *Human Gene Therapy* 6:185-193; and Kaplitt (1994) *Nature Genetics* 6:148-153.

Retroviral vectors are well known in the art and we contemplate that any retroviral gene therapy vector is employable in the invention, including B, C and D type retroviruses, xenotropic retroviruses (for example, NZB-X1, NZB-X2 and NZB9-1 (see O'Neill (1985) *J. Virol.* 53:160) polytropic retroviruses *e.g.* MCF and MCF-MLV (see Kelly (1983) *J. Virol.* 45:291), spumaviruses and lentiviruses. See RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985.

Portions of the retroviral gene therapy vector may be derived from different retroviruses. For example, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

These recombinant retroviral vectors may be used to generate transduction competent retroviral vector particles by introducing them into appropriate packaging cell lines (see US patent 5,591,624). Retrovirus vectors can be constructed for site-specific integration into host cell DNA by incorporation of a chimeric integrase enzyme into the retroviral particle (see WO96/37626). It is preferable that the recombinant viral vector is a replication defective recombinant virus.

Packaging cell lines suitable for use with the above-described retrovirus vectors are well known in the art, are readily prepared (see WO95/30763 and WO92/05266), and can be used to create producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant vector particles. Preferably, the packaging cell lines are made from human parent cells (*e.g.* HT1080 cells) or mink parent cell lines, which eliminates inactivation in human serum.

Preferred retroviruses for the construction of retroviral gene therapy vectors include Avian Leukosis Virus, Bovine Leukemia, Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis Virus and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe (1976) *J Virol* 19:19-25), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC Nol VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998) and Moloney Murine Leukemia Virus (ATCC No. VR-190). Such retroviruses may be obtained from depositories or collections such as the American Type Culture Collection ("ATCC") in Rockville, Maryland or isolated from known sources using commonly available techniques.

Exemplary known retroviral gene therapy vectors employable in this invention include those described in patent applications GB2200651, EP0415731, EP0345242, EP0334301, WO89/02468; WO89/05349, WO89/09271, WO90/02806, WO90/07936, WO94/03622, WO93/25698, WO93/25234, WO93/11230, WO93/10218, WO91/02805, WO91/02825, WO95/07994, US 5,219,740, US 4,405,712, US 4,861,719, US 4,980,289, US 4,777,127, US 5,591,624. See also Vile (1993) *Cancer Res* 53:3860-3864; Vile (1993) *Cancer Res* 53:962-967; Ram (1993) *Cancer Res* 53 (1993) 83-88; Takamiya (1992) *J Neurosci Res* 33:493-503; Baba (1993) *J Neurosurg* 79:729-735; Mann (1983) *Cell* 33:153; Cane (1984) *Proc Natl Acad Sci* 81:6349; and Miller (1990) *Human Gene Therapy* 1.

Human adenoviral gene therapy vectors are also known in the art and employable in this invention. See, for example, Berkner (1988) *Biotechniques* 6:616 and Rosenfeld (1991) *Science* 252:431, and WO93/07283, WO93/06223, and WO93/07282. Exemplary known adenoviral gene therapy vectors employable in this invention include those described in the above referenced documents and in WO94/12649, WO93/03769, WO93/19191, WO94/28938, WO95/11984, WO95/00655, WO95/27071, WO95/29993, WO95/34671,

WO96/05320, WO94/08026, WO94/11506, WO93/06223, WO94/24299, WO95/14102, WO95/24297, WO95/02697, WO94/28152, WO94/24299, WO95/09241, WO95/25807, WO95/05835, WO94/18922 and WO95/09654. Alternatively, administration of DNA linked to killed adenovirus as described in Curiel (1992) *Hum. Gene Ther.* 3:147-154 may be employed. The gene delivery vehicles of the invention also include adenovirus associated virus (AAV) vectors. Leading and preferred examples of such vectors for use in this invention are the AAV-2 based vectors disclosed in Srivastava, WO93/09239. Most preferred AAV vectors comprise the two AAV inverted terminal repeats in which the native D-sequences are modified by substitution of nucleotides, such that at least 5 native nucleotides and up to 18 native nucleotides, preferably at least 10 native nucleotides up to 18 native nucleotides, most preferably 10 native nucleotides are retained and the remaining nucleotides of the D-sequence are deleted or replaced with non-native nucleotides. The native D-sequences of the AAV inverted terminal repeats are sequences of 20 consecutive nucleotides in each AAV inverted terminal repeat (*i.e.* there is one sequence at each end) which are not involved in HP formation. The non-native replacement nucleotide may be any nucleotide other than the nucleotide found in the native D-sequence in the same position. Other employable exemplary AAV vectors are pWP-19, pWN-1, both of which are disclosed in Nahreini (1993) *Gene* 124:257-262. Another example of such an AAV vector is psub201 (see Samulski (1987) *J. Virol.* 61:3096). Another exemplary AAV vector is the Double-D ITR vector. Construction of the Double-D ITR vector is disclosed in US Patent 5,478,745. Still other vectors are those disclosed in Carter US Patent 4,797,368 and Muzyczka US Patent 5,139,941, Chartejee US Patent 5,474,935, and Kotin WO94/288157. Yet a further example of an AAV vector employable in this invention is SSV9AFABTKneo, which contains the AFP enhancer and albumin promoter and directs expression predominantly in the liver. Its structure and construction are disclosed in Su (1996) *Human Gene Therapy* 7:463-470. Additional AAV gene therapy vectors are described in US 5,354,678, US 5,173,414, US 5,139,941, and US 5,252,479.

The gene therapy vectors of the invention also include herpes vectors. Leading and preferred examples are herpes simplex virus vectors containing a sequence encoding a thymidine kinase polypeptide such as those disclosed in US 5,288,641 and EP0176170 (Roizman). Additional exemplary herpes simplex virus vectors include HFEM/ICP6-LacZ disclosed in WO95/04139 (Wistar Institute), pHSVlac described in Geller (1988) *Science* 241:1667-1669 and in WO90/09441 and WO92/07945, HSV Us3::pgC-lacZ described in Fink (1992) *Human Gene Therapy* 3:11-19 and HSV 7134, 2 RH 105 and GAL4 described in EP 0453242 (Breakefield), and those deposited with the ATCC as accession numbers ATCC VR-977 and ATCC VR-260.

Also contemplated are alpha virus gene therapy vectors that can be employed in this invention. Preferred alpha virus vectors are Sindbis viruses vectors. Togaviruses, Semliki Forest virus (ATCC VR-67; ATCC VR-1247), Middleberg virus (ATCC VR-370), Ross River virus (ATCC VR-373; ATCC VR-1246), Venezuelan equine encephalitis virus (ATCC VR923; ATCC VR-1250; ATCC VR-1249; ATCC VR-532), and those described in US patents 5,091,309, 5,217,879, and WO92/10578. More particularly, those alpha

virus vectors described in US Serial No. 08/405,627, filed March 15, 1995,WO94/21792, WO92/10578, WO95/07994, US 5,091,309 and US 5,217,879 are employable. Such alpha viruses may be obtained from depositories or collections such as the ATCC in Rockville, Maryland or isolated from known sources using commonly available techniques. Preferably, alphavirus vectors with reduced cytotoxicity are used (see USSN 08/679640).

DNA vector systems such as eukaryotic layered expression systems are also useful for expressing the nucleic acids of the invention. See WO95/07994 for a detailed description of eukaryotic layered expression systems. Preferably, the eukaryotic layered expression systems of the invention are derived from alphavirus vectors and most preferably from Sindbis viral vectors.

Other viral vectors suitable for use in the present invention include those derived from poliovirus, for example ATCC VR-58 and those described in Evans, Nature 339 (1989) 385 and Sabin (1973) *J. Biol. Standardization* 1:115; rhinovirus, for example ATCC VR-1110 and those described in Arnold (1990) *J Cell Biochem* L401; pox viruses such as canary pox virus or vaccinia virus, for example ATCC VR-111 and ATCC VR-2010 and those described in Fisher-Hoch (1989) *Proc Natl Acad Sci* 86:317; Flexner (1989) *Ann NY Acad Sci* 569:86, Flexner (1990) *Vaccine* 8:17; in US 4,603,112 and US 4,769,330 and WO89/01973; SV40 virus, for example ATCC VR-305 and those described in Mulligan (1979) *Nature* 277:108 and Madzak (1992) *J Gen Virol* 73:1533; influenza virus, for example ATCC VR-797 and recombinant influenza viruses made employing reverse genetics techniques as described in US 5,166,057 and in Enami (1990) *Proc Natl Acad Sci* 87:3802-3805; Enami & Palese (1991) *J Virol* 65:2711-2713 and Luytjes (1989) *Cell* 59:110, (see also McMichael (1983) *NEJ Med* 309:13, and Yap (1978) *Nature* 273:238 and *Nature* (1979) 277:108); human immunodeficiency virus as described in EP-0386882 and in Buchschacher (1992) *J. Virol.* 66:2731; measles virus, for example ATCC VR-67 and VR-1247 and those described in EP-0440219; Aura virus, for example ATCC VR-368; Bebaru virus, for example ATCC VR-600 and ATCC VR-1240; Cabassou virus, for example ATCC VR-922; Chikungunya virus, for example ATCC VR-64 and ATCC VR-1241; Fort Morgan Virus, for example ATCC VR-924; Getah virus, for example ATCC VR-369 and ATCC VR-1243; Kyzylagach virus, for example ATCC VR-927; Mayaro virus, for example ATCC VR-66; Mucambo virus, for example ATCC VR-580 and ATCC VR-1244; Ndumu virus, for example ATCC VR-371; Pixuna virus, for example ATCC VR-372 and ATCC VR-1245; Tonate virus, for example ATCC VR-925; Triniti virus, for example ATCC VR-469; Una virus, for example ATCC VR-374; Whataroa virus, for example ATCC VR-926; Y-62-33 virus, for example ATCC VR-375; O'Nyong virus, Eastern encephalitis virus, for example ATCC VR-65 and ATCC VR-1242; Western encephalitis virus, for example ATCC VR-70, ATCC VR-1251, ATCC VR-622 and ATCC VR-1252; and coronavirus, for example ATCC VR-740 and those described in Hamre (1966) *Proc Soc Exp Biol Med* 121:190.

Delivery of the compositions of this invention into cells is not limited to the above mentioned viral vectors. Other delivery methods and media may be employed such as, for example, nucleic acid expression vectors,

polycationic condensed DNA linked or unlinked to killed adenovirus alone, for example see US Serial No. 08/366,787, filed December 30, 1994 and Curiel (1992) *Hum Gene Ther* 3:147-154 ligand linked DNA, for example see Wu (1989) *J Biol Chem* 264:16985-16987, eucaryotic cell delivery vehicles cells, for example see US Serial No.08/240,030, filed May 9, 1994, and US Serial No. 08/404,796, deposition of photopolymerized hydrogel materials, hand-held gene transfer particle gun, as described in US Patent 5,149,655, ionizing radiation as described in US5,206,152 and in WO92/11033, nucleic charge neutralization or fusion with cell membranes. Additional approaches are described in Philip (1994) *Mol Cell Biol* 14:2411-2418 and in Woffendin (1994) *Proc Natl Acad Sci* 91:1581-1585.

Particle mediated gene transfer may be employed, for example see US Serial No. 60/023,867. Briefly, the sequence can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, as described in Wu & Wu (1987) *J. Biol. Chem.* 262:4429-4432, insulin as described in Hucked (1990) *Biochem Pharmacol* 40:253-263, galactose as described in Plank (1992) *Bioconjugate Chem* 3:533-539, lactose or transferrin.

Naked DNA may also be employed. Exemplary naked DNA introduction methods are described in WO 90/11092 and US 5,580,859. Uptake efficiency may be improved using biodegradable latex beads. DNA coated latex beads are efficiently transported into cells after endocytosis initiation by the beads. The method may be improved further by treatment of the beads to increase hydrophobicity and thereby facilitate disruption of the endosome and release of the DNA into the cytoplasm.

Liposomes that can act as gene delivery vehicles are described in US 5,422,120, WO95/13796, WO94/23697, WO91/14445 and EP-524,968. As described in USSN. 60/023,867, on non-viral delivery, the nucleic acid sequences encoding a polypeptide can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then be incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, insulin, galactose, lactose, or transferrin. Other delivery systems include the use of liposomes to encapsulate DNA comprising the gene under the control of a variety of tissue-specific or ubiquitously-active promoters. Further non-viral delivery suitable for use includes mechanical delivery systems such as the approach described in Woffendin *et al* (1994) *Proc. Natl. Acad. Sci. USA* 91(24):11581-11585. Moreover, the coding sequence and the product of expression of such can be delivered through deposition of photopolymerized hydrogel materials. Other conventional methods for gene delivery that can be used for delivery of the coding sequence include, for example, use of hand-held gene transfer particle gun, as described in US 5,149,655; use of ionizing radiation for activating transferred gene, as described in US 5,206,152 and WO92/11033

Exemplary liposome and polycationic gene delivery vehicles are those described in US 5,422,120 and 4,762,915; in WO 95/13796; WO94/23697; and WO91/14445; in EP-0524968; and in Stryer, Biochemistry, pages 236-240 (1975) W.H. Freeman, San Francisco; Szoka (1980) *Biochem Biophys Acta* 600:1; Bayer (1979) *Biochem Biophys Acta* 550:464; Rivnay (1987) *Meth Enzymol* 149:119; Wang (1987) *Proc Natl Acad Sci* 84:7851; Plant (1989) *Anal Biochem* 176:420.

A polynucleotide composition can comprises therapeutically effective amount of a gene therapy vehicle, as the term is defined above. For purposes of the present invention, an effective dose will be from about 0.01 mg/ kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of the DNA constructs in the individual to which it is administered.

Delivery Methods

Once formulated, the polynucleotide compositions of the invention can be administered (1) directly to the subject; (2) delivered *ex vivo*, to cells derived from the subject; or (3) *in vitro* for expression of recombinant proteins. The subjects to be treated can be mammals or birds. Also, human subjects can be treated.

Direct delivery of the compositions will generally be accomplished by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly or delivered to the interstitial space of a tissue. The compositions can also be administered into a lesion. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal or transcutaneous applications (*e.g.* see WO98/20734), needles, and gene guns or hyposprays. Dosage treatment may be a single dose schedule or a multiple dose schedule.

Methods for the *ex vivo* delivery and reimplantation of transformed cells into a subject are known in the art and described in *e.g.* WO93/14778. Examples of cells useful in ex vivo applications include, for example, stem cells, particularly hematopoetic, lymph cells, macrophages, dendritic cells, or tumor cells.

Generally, delivery of nucleic acids for both *ex vivo* and *in vitro* applications can be accomplished by the following procedures, for example, dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei, all well known in the art.

Polynucleotide and polypeptide pharmaceutical compositions

In addition to the pharmaceutically acceptable carriers and salts described above, the following additional agents can be used with polynucleotide and/or polypeptide compositions.

A.Polypeptides

One example are polypeptides which include, without limitation: asioloorosomucoid (ASOR); transferrin; asialoglycoproteins; antibodies; antibody fragments; ferritin; interleukins; interferons, granulocyte, macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), stem cell factor and erythropoietin. Viral antigens, such as

envelope proteins, can also be used. Also, proteins from other invasive organisms, such as the 17 amino acid peptide from the circumsporozoite protein of plasmodium falciparum known as RII.

B.Hormones, Vitamins, *etc.*

Other groups that can be included are, for example: hormones, steroids, androgens, estrogens, thyroid hormone, or vitamins, folic acid.

C.Polyalkylenes, Polysaccharides, *etc.*

Also, polyalkylene glycol can be included with the desired polynucleotides/polypeptides. In a preferred embodiment, the polyalkylene glycol is polyethlylene glycol. In addition, mono-, di-, or polysaccharides can be included. In a preferred embodiment of this aspect, the polysaccharide is dextran or DEAE-dextran. Also, chitosan and poly(lactide-co-glycolide)

D.Lipids, and Liposomes

The desired polynucleotide/polypeptide can also be encapsulated in lipids or packaged in liposomes prior to delivery to the subject or to cells derived therefrom.

Lipid encapsulation is generally accomplished using liposomes which are able to stably bind or entrap and retain nucleic acid. The ratio of condensed polynucleotide to lipid preparation can vary but will generally be around 1:1 (mg DNA:micromoles lipid), or more of lipid. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight (1991) *Biochim. Biophys. Acta.* 1097:1-17; Straubinger (1983) *Meth. Enzymol.* 101:512-527.

Liposomal preparations for use in the present invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner (1987) *Proc. Natl. Acad. Sci. USA* 84:7413-7416); mRNA (Malone (1989) *Proc. Natl. Acad. Sci. USA* 86:6077-6081); and purified transcription factors (Debs (1990) *J. Biol. Chem.* 265:10189-10192), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. (See, also, Felgner *supra*). Other commercially available liposomes include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boerhinger). Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, *e.g.* Szoka (1978) *Proc. Natl. Acad. Sci. USA* 75:4194-4198; WO90/11092 for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphoshatidyl ethanolamine (DOPE), among others. These

materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

The liposomes can comprise multilammelar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs). The various liposome-nucleic acid complexes are prepared using methods known in the art. See *e.g.* Straubinger (1983) *Meth. Immunol.* 101:512-527; Szoka (1978) *Proc. Natl. Acad. Sci. USA* 75:4194-4198; Papahadjopoulos (1975) *Biochim. Biophys. Acta* 394:483; Wilson (1979) *Cell* 17:77); Deamer & Bangham (1976) *Biochim. Biophys. Acta* 443:629; Ostro (1977) *Biochem. Biophys. Res. Commun.* 76:836; Fraley (1979) *Proc. Natl. Acad. Sci. USA* 76:3348); Enoch & Strittmatter (1979) *Proc. Natl. Acad. Sci. USA* 76:145; Fraley (1980) *J. Biol. Chem.* (1980) 255:10431; Szoka & Papahadjopoulos (1978) *Proc. Natl. Acad. Sci. USA* 75:145; and Schaefer-Ridder (1982) *Science* 215:166.

E.Lipoproteins

In addition, lipoproteins can be included with the polynucleotide/polypeptide to be delivered. Examples of lipoproteins to be utilized include: chylomicrons, HDL, IDL, LDL, and VLDL. Mutants, fragments, or fusions of these proteins can also be used. Also, modifications of naturally occurring lipoproteins can be used, such as acetylated LDL. These lipoproteins can target the delivery of polynucleotides to cells expressing lipoprotein receptors. Preferably, if lipoproteins are including with the polynucleotide to be delivered, no other targeting ligand is included in the composition.

Naturally occurring lipoproteins comprise a lipid and a protein portion. The protein portion are known as apoproteins. At the present, apoproteins A, B, C, D, and E have been isolated and identified. At least two of these contain several proteins, designated by Roman numerals, AI, AII, AIV; CI, CII, CIII.

A lipoprotein can comprise more than one apoprotein. For example, naturally occurring chylomicrons comprises of A, B, C & E, over time these lipoproteins lose A and acquire C & E apoproteins. VLDL comprises A, B, C & E apoproteins, LDL comprises apoprotein B; HDL comprises apoproteins A, C, & E.

The amino acid of these apoproteins are known and are described in, for example, Breslow (1985) Annu Rev. Biochem 54:699; Law (1986) Adv. Exp Med. Biol. 151:162; Chen (1986) J Biol Chem 261:12918; Kane (1980) Proc Natl Acad Sci USA 77:2465; and Utermann (1984) Hum Genet 65:232.

Lipoproteins contain a variety of lipids including, triglycerides, cholesterol (free and esters), and phospholipids. The composition of the lipids varies in naturally occurring lipoproteins. For example, chylomicrons comprise mainly triglycerides. A more detailed description of the lipid content of naturally occurring lipoproteins can be found, for example, in *Meth. Enzymol.* 128 (1986). The composition of the lipids are chosen to aid in conformation of the apoprotein for receptor binding activity. The composition of lipids can also be chosen to facilitate hydrophobic interaction and association with the polynucleotide binding molecule.

Naturally occurring lipoproteins can be isolated from serum by ultracentrifugation, for instance. Such methods are described in *Meth. Enzymol. (supra)*; Pitas (1980) *J. Biochem.* 255:5454-5460 and Mahey (1979) *J Clin. Invest* 64:743-750. Lipoproteins can also be produced by *in vitro* or recombinant methods by expression of the apoprotein genes in a desired host cell. See, for example, Atkinson (1986) *Annu Rev Biophys Chem* 15:403 and Radding (1958) *Biochim Biophys Acta* 30: 443. Lipoproteins can also be purchased from commercial suppliers, such as Biomedical Techniologies, Inc., Stoughton, Massachusetts, USA. Further description of lipoproteins can be found in Zuckermann *et al.* WO98/06437.

F.Polycationic Agents

Polycationic agents can be included, with or without lipoprotein, in a composition with the desired polynucleotide/polypeptide to be delivered.

Polycationic agents, typically, exhibit a net positive charge at physiological relevant pH and are capable of neutralizing the electrical charge of nucleic acids to facilitate delivery to a desired location. These agents have both in vitro, ex vivo, and in vivo applications. Polycationic agents can be used to deliver nucleic acids to a living subject either intramuscularly, subcutaneously, etc.

The following are examples of useful polypeptides as polycationic agents: polylysine, polyarginine, polyornithine, and protamine. Other examples include histones, protamines, human serum albumin, DNA binding proteins, non-histone chromosomal proteins, coat proteins from DNA viruses, such as (X174, transcriptional factors also contain domains that bind DNA and therefore may be useful as nucleic aid condensing agents. Briefly, transcriptional factors such as C/CEBP, c-jun, c-fos, AP-1, AP-2, AP-3, CPF, Prot-1, Sp-1, Oct-1, Oct-2, CREP, and TFIID contain basic domains that bind DNA sequences.

Organic polycationic agents include: spermine, spermidine, and purtrescine.

The dimensions and of the physical properties of a polycationic agent can be extrapolated from the list above, to construct other polypeptide polycationic agents or to produce synthetic polycationic agents.

Synthetic polycationic agents which are useful include, for example, DEAE-dextran, polybrene. Lipofectin™, and lipofectAMINE™ are monomers that form polycationic complexes when combined with polynucleotides/polypeptides.

Immunodiagnostic Assays

Meningogoccal antigens of the invention can be used in immunoassays to detect antibody levels (or, conversely, anti-meningococcal antibodies can be used to detect antigen levels). Immunoassays based on well defined, recombinant antigens can be developed to replace invasive diagnostics methods. Antibodies to meningococcal proteins within biological samples, including for example, blood or serum samples, can be detected. Design of the immunoassays is subject to a great deal of variation, and a variety of these are known in the art. Protocols for the immunoassay may be based, for example, upon competition, or direct reaction, or sandwich type assays. Protocols may also, for example, use solid supports, or may be by

immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be, for example, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the probe are also known; examples of which are assays which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

Kits suitable for immunodiagnosis and containing the appropriate labeled reagents are constructed by packaging the appropriate materials, including the compositions of the invention, in suitable containers, along with the remaining reagents and materials (for example, suitable buffers, salt solutions, *etc.*) required for the conduct of the assay, as well as suitable set of assay instructions.

Nucleic Acid Hybridisation

"Hybridization" refers to the association of two nucleic acid sequences to one another by hydrogen bonding. Typically, one sequence will be fixed to a solid support and the other will be free in solution. Then, the two sequences will be placed in contact with one another under conditions that favor hydrogen bonding. Factors that affect this bonding include: the type and volume of solvent; reaction temperature; time of hybridization; agitation; agents to block the non-specific attachment of the liquid phase sequence to the solid support (Denhardt's reagent or BLOTTO); concentration of the sequences; use of compounds to increase the rate of association of sequences (dextran sulfate or polyethylene glycol); and the stringency of the washing conditions following hybridization. See Sambrook *et al.* [*supra*] Volume 2, chapter 9, pages 9.47 to 9.57.

"Stringency" refers to conditions in a hybridization reaction that favor association of very similar sequences over sequences that differ. For example, the combination of temperature and salt concentration should be chosen that is approximately 120 to 200°C below the calculated Tm of the hybrid under study. The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the sequence of interest and then washed under conditions of different stringencies. See Sambrook *et al.* at page 9.50.

Variables to consider when performing, for example, a Southern blot are (1) the complexity of the DNA being blotted and (2) the homology between the probe and the sequences being detected. The total amount of the fragment(s) to be studied can vary a magnitude of 10, from 0.1 to 1µg for a plasmid or phage digest to 10^{-9} to 10^{-8} g for a single copy gene in a highly complex eukaryotic genome. For lower complexity polynucleotides, substantially shorter blotting, hybridization, and exposure times, a smaller amount of starting polynucleotides, and lower specific activity of probes can be used. For example, a single-copy yeast gene can be detected with an exposure time of only 1 hour starting with 1 µg of yeast DNA, blotting for two hours, and hybridizing for 4-8 hours with a probe of 10^8 cpm/µg. For a single-copy mammalian gene a conservative approach would start with 10 µg of DNA, blot overnight, and hybridize overnight in the presence of 10% dextran sulfate using a probe of greater than 10^8 cpm/µg, resulting in an exposure time of ~24 hours.

Several factors can affect the melting temperature (Tm) of a DNA-DNA hybrid between the probe and the fragment of interest, and consequently, the appropriate conditions for hybridization and washing. In many cases the probe is not 100% homologous to the fragment. Other commonly encountered variables include the length and total G+C content of the hybridizing sequences and the ionic strength and formamide content of the hybridization buffer. The effects of all of these factors can be approximated by a single equation:

$$Tm = 81 + 16.6(\log_{10}Ci) + 0.4[\%(G + C)] - 0.6(\%formamide) - 600/n - 1.5(\%mismatch).$$

where Ci is the salt concentration (monovalent ions) and *n* is the length of the hybrid in base pairs (slightly modified from Meinkoth & Wahl (1984) *Anal. Biochem.* 138: 267-284).

In designing a hybridization experiment, some factors affecting nucleic acid hybridization can be conveniently altered. The temperature of the hybridization and washes and the salt concentration during the washes are the simplest to adjust. As the temperature of the hybridization increases (*i.e.* stringency), it becomes less likely for hybridization to occur between strands that are nonhomologous, and as a result, background decreases. If the radiolabeled probe is not completely homologous with the immobilized fragment (as is frequently the case in gene family and interspecies hybridization experiments), the hybridization temperature must be reduced, and background will increase. The temperature of the washes affects the intensity of the hybridizing band and the degree of background in a similar manner. The stringency of the washes is also increased with decreasing salt concentrations.

In general, convenient hybridization temperatures in the presence of 50% formamide are 42°C for a probe with is 95% to 100% homologous to the target fragment, 37°C for 90% to 95% homology, and 32°C for 85% to 90% homology. For lower homologies, formamide content should be lowered and temperature adjusted accordingly, using the equation above. If the homology between the probe and the target fragment are not known, the simplest approach is to start with both hybridization and wash conditions which are nonstringent. If non-specific bands or high background are observed after autoradiography, the filter can be washed at high stringency and reexposed. If the time required for exposure makes this approach impractical, several hybridization and/or washing stringencies should be tested in parallel.

Nucleic Acid Probe Assays

Methods such as PCR, branched DNA probe assays, or blotting techniques utilizing nucleic acid probes according to the invention can determine the presence of cDNA or mRNA. A probe is said to "hybridize" with a sequence of the invention if it can form a duplex or double stranded complex, which is stable enough to be detected.

The nucleic acid probes will hybridize to the meningococcal nucleotide sequences of the invention (including both sense and antisense strands). Though many different nucleotide sequences will encode the amino acid sequence, the native meningococcal sequence is preferred because it is the actual sequence present in cells. mRNA represents a coding sequence and so a probe should be complementary to the coding

sequence; single-stranded cDNA is complementary to mRNA, and so a cDNA probe should be complementary to the non-coding sequence.

The probe sequence need not be identical to the meningococcal sequence (or its complement) — some variation in the sequence and length can lead to increased assay sensitivity if the nucleic acid probe can form a duplex with target nucleotides, which can be detected. Also, the nucleic acid probe can include additional nucleotides to stabilize the formed duplex. Additional meningococcal sequence may also be helpful as a label to detect the formed duplex. For example, a non-complementary nucleotide sequence may be attached to the 5' end of the probe, with the remainder of the probe sequence being complementary to a meningococcal sequence. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the a meningococcal sequence in order to hybridize therewith and thereby form a duplex which can be detected.

The exact length and sequence of the probe will depend on the hybridization conditions, such as temperature, salt condition and the like. For example, for diagnostic applications, depending on the complexity of the analyte sequence, the nucleic acid probe typically contains at least 10-20 nucleotides, preferably 15-25, and more preferably at least 30 nucleotides, although it may be shorter than this. Short primers generally require cooler temperatures to form sufficiently stable hybrid complexes with the template.

Probes may be produced by synthetic procedures, such as the triester method of Matteucci *et al.* [*J. Am. Chem. Soc.* (1981) 103:3185], or according to Urdea *et al.* [*Proc. Natl. Acad. Sci. USA* (1983) 80: 7461], or using commercially available automated oligonucleotide synthesizers.

The chemical nature of the probe can be selected according to preference. For certain applications, DNA or RNA are appropriate. For other applications, modifications may be incorporated *e.g.* backbone modifications, such as phosphorothioates or methylphosphonates, can be used to increase *in vivo* half-life, alter RNA affinity, increase nuclease resistance *etc.* [*e.g.* see Agrawal & Iyer (1995) *Curr Opin Biotechnol* 6:12-19; Agrawal (1996) *TIBTECH* 14:376-387]; analogues such as peptide nucleic acids may also be used [*e.g.* see Corey (1997) *TIBTECH* 15:224-229; Buchardt *et al.* (1993) *TIBTECH* 11:384-386].

Alternatively, the polymerase chain reaction (PCR) is another well-known means for detecting small amounts of target nucleic acids. The assay is described in: Mullis *et al.* [*Meth. Enzymol.* (1987) 155: 335-350]; US patents 4,683,195 and 4,683,202. Two "primer" nucleotides hybridize with the target nucleic acids and are used to prime the reaction. The primers can comprise sequence that does not hybridize to the sequence of the amplification target (or its complement) to aid with duplex stability or, for example, to incorporate a convenient restriction site. Typically, such sequence will flank the desired meningococcal sequence.

A thermostable polymerase creates copies of target nucleic acids from the primers using the original target nucleic acids as a template. After a threshold amount of target nucleic acids are generated by the polymerase,

they can be detected by more traditional methods, such as Southern blots. When using the Southern blot method, the labelled probe will hybridize to the meningococcal sequence (or its complement).

Also, mRNA or cDNA can be detected by traditional blotting techniques described in Sambrook *et al* [*supra*]. mRNA, or cDNA generated from mRNA using a polymerase enzyme, can be purified and separated using gel electrophoresis. The nucleic acids on the gel are then blotted onto a solid support, such as nitrocellulose. The solid support is exposed to a labelled probe and then washed to remove any unhybridized probe. Next, the duplexes containing the labeled probe are detected. Typically, the probe is labelled with a radioactive moiety.

EXAMPLES OF PREFERRED FRAGMENTS

The protein sequences disclosed in WO99/36544 have been subjected to computer analysis to predict antigenic peptide fragments within the full-length proteins. Three algorithms have been used in this analysis:

- **AMPHI** This program has been used to predict T-cell epitopes [Gao *et al.* (1989) *J. Immunol.* 143:3007; Roberts *et al.* (1996) *AIDS Res Hum Retrovir* 12:593; Quakyi *et al.* (1992) *Scand J Immunol* suppl.11:9] and is available in the Protean package of DNASTAR, Inc. (1228 South Park Street, Madison, Wisconsin 53715 USA).

- **ANTIGENIC INDEX** as disclosed by Jameson & Wolf (1988) The antigenic index: a novel algorithm for predicting antigenic determinants. *CABIOS* 4:181:186.

- **HYDROPHILICITY** as disclosed by Hopp & Woods (1981) Prediction of protein antigenic determinants from amino acid sequences. *PNAS USA* 78:3824-3828

Table I indicates preferred fragments of the proteins disclosed in WO99/36544. The three algorithms often identify the same fragments (*e.g.* ORF38-1 – the fragments from residue 37-42 and 143-146 are both identified twice). Such multiply-identified fragments are particularly preferred. The algorithms often identify overlapping fragments (*e.g.* ORF40-1 – AMPHI identifies residues 161-165, and Hydrophilicity identified residues 163-175). The invention explicitly includes fragments resulting from a combination of these overlapping fragments (*e.g.* the fragment from residue 161 to residue 175 in the case of ORF40-1). Fragments separated by a single amino acid are also often identified (*e.g.* ORF40-1 Antigenic Index 423-426 and 428-438). The invention also includes fragments spanning the two extremes of such "adjacent" fragments (*e.g.* 423-438 for ORF40-1).

TABLE I – 1769 fragments of the proteins disclosed in WO99/36544

Key: fragment#1 of the present application is amino acids 6-14 of ORF38-1 disclosed in WO99/36544, fragment#2 of the present application is amino acids 57-59 of ORF38-1 disclosed in WO99/36544 *etc.*

Fragment#	WO99/36544 ORF	Algorithm	Amino acids
1.	38-1	AMPHI	6-14
2.	38-1	AMPHI	57-59
3.	38-1	AMPHI	67-76
4.	38-1	AMPHI	92-100
5.	38-1	AMPHI	127-137
6.	38-1	AMPHI	149-166
7.	38-1	AMPHI	210-215
8.	38-1	AMPHI	231-236
9.	38-1	AMPHI	270-272
10.	38-1	AMPHI	303-320
11.	38-1	Antigenic Index	16-34
12.	38-1	Antigenic Index	37-42
13.	38-1	Antigenic Index	46-64
14.	38-1	Antigenic Index	72-91
15.	38-1	Antigenic Index	94-112
16.	38-1	Antigenic Index	114-117
17.	38-1	Antigenic Index	124-136
18.	38-1	Antigenic Index	143-146
19.	38-1	Antigenic Index	148-160
20.	38-1	Antigenic Index	167-195
21.	38-1	Antigenic Index	201-216
22.	38-1	Antigenic Index	218-240
23.	38-1	Antigenic Index	244-252
24.	38-1	Antigenic Index	257-278
25.	38-1	Antigenic Index	282-290
26.	38-1	Antigenic Index	308-314
27.	38-1	Hydrophilicity	21-34
28.	38-1	Hydrophilicity	37-42
29.	38-1	Hydrophilicity	47-55
30.	38-1	Hydrophilicity	57-61
31.	38-1	Hydrophilicity	72-74
32.	38-1	Hydrophilicity	76-78
33.	38-1	Hydrophilicity	82-91
34.	38-1	Hydrophilicity	94-101
35.	38-1	Hydrophilicity	108-112
36.	38-1	Hydrophilicity	126-136
37.	38-1	Hydrophilicity	143-146
38.	38-1	Hydrophilicity	148-160

39.	38-1	Hydrophilicity	167-195
40.	38-1	Hydrophilicity	221-223
41.	38-1	Hydrophilicity	226-236
42.	38-1	Hydrophilicity	244-250
43.	38-1	Hydrophilicity	257-274
44.	38-1	Hydrophilicity	282-286
45.	38-1	Hydrophilicity	311-314
46.	38a	AMPHI	6-14
47.	38a	AMPHI	57-59
48.	38a	AMPHI	67-76
49.	38a	AMPHI	92-100
50.	38a	AMPHI	127-137
51.	38a	AMPHI	149-166
52.	38a	AMPHI	210-215
53.	38a	AMPHI	223-225
54.	38a	AMPHI	231-236
55.	38a	AMPHI	270-272
56.	38a	AMPHI	303-320
57.	38a	Antigenic Index	16-34
58.	38a	Antigenic Index	37-42
59.	38a	Antigenic Index	46-64
60.	38a	Antigenic Index	72-91
61.	38a	Antigenic Index	94-112
62.	38a	Antigenic Index	114-117
63.	38a	Antigenic Index	124-136
64.	38a	Antigenic Index	143-146
65.	38a	Antigenic Index	148-160
66.	38a	Antigenic Index	165-195
67.	38a	Antigenic Index	201-216
68.	38a	Antigenic Index	218-240
69.	38a	Antigenic Index	244-252
70.	38a	Antigenic Index	257-278
71.	38a	Antigenic Index	282-290
72.	38a	Antigenic Index	308-314
73.	38a	Hydrophilicity	21-34
74.	38a	Hydrophilicity	37-42
75.	38a	Hydrophilicity	47-55
76.	38a	Hydrophilicity	57-61
77.	38a	Hydrophilicity	72-74
78.	38a	Hydrophilicity	76-78
79.	38a	Hydrophilicity	82-91
80.	38a	Hydrophilicity	94-101
81.	38a	Hydrophilicity	108-112
82.	38a	Hydrophilicity	126-136
83.	38a	Hydrophilicity	143-146

84.	38a	Hydrophilicity	148-160
85.	38a	Hydrophilicity	165-195
86.	38a	Hydrophilicity	221-223
87.	38a	Hydrophilicity	226-236
88.	38a	Hydrophilicity	244-250
89.	38a	Hydrophilicity	257-273
90.	38a	Hydrophilicity	282-286
91.	38a	Hydrophilicity	311-314
92.	39-1	AMPHI	6-13
93.	39-1	AMPHI	21-24
94.	39-1	AMPHI	37-40
95.	39-1	AMPHI	60-75
96.	39-1	AMPHI	118-122
97.	39-1	AMPHI	134-139
98.	39-1	AMPHI	165-183
99.	39-1	AMPHI	192-195
100.	39-1	AMPHI	233-241
101.	39-1	AMPHI	247-267
102.	39-1	AMPHI	273-275
103.	39-1	AMPHI	299-308
104.	39-1	AMPHI	310-319
105.	39-1	AMPHI	322-330
106.	39-1	AMPHI	338-347
107.	39-1	AMPHI	358-364
108.	39-1	AMPHI	366-368
109.	39-1	AMPHI	376-378
110.	39-1	AMPHI	385-392
111.	39-1	AMPHI	413-416
112.	39-1	AMPHI	421-424
113.	39-1	AMPHI	429-438
114.	39-1	AMPHI	445-454
115.	39-1	AMPHI	456-458
116.	39-1	AMPHI	498-500
117.	39-1	AMPHI	512-519
118.	39-1	AMPHI	576-587
119.	39-1	AMPHI	589-600
120.	39-1	AMPHI	650-652
121.	39-1	AMPHI	670-674
122.	39-1	Antigenic Index	26-32
123.	39-1	Antigenic Index	35-45
124.	39-1	Antigenic Index	54-69
125.	39-1	Antigenic Index	79-84
126.	39-1	Antigenic Index	88-96
127.	39-1	Antigenic Index	105-110
128.	39-1	Antigenic Index	117-124

129.	39-1	Antigenic Index	152-154
130.	39-1	Antigenic Index	190-192
131.	39-1	Antigenic Index	222-231
132.	39-1	Antigenic Index	246-265
133.	39-1	Antigenic Index	292-295
134.	39-1	Antigenic Index	318-335
135.	39-1	Antigenic Index	353-362
136.	39-1	Antigenic Index	370-372
137.	39-1	Antigenic Index	402-404
138.	39-1	Antigenic Index	406-408
139.	39-1	Antigenic Index	419-421
140.	39-1	Antigenic Index	446-449
141.	39-1	Antigenic Index	453-460
142.	39-1	Antigenic Index	465-469
143.	39-1	Antigenic Index	476-487
144.	39-1	Antigenic Index	491-499
145.	39-1	Antigenic Index	505-514
146.	39-1	Antigenic Index	522-536
147.	39-1	Antigenic Index	557-567
148.	39-1	Antigenic Index	569-575
149.	39-1	Antigenic Index	577-580
150.	39-1	Antigenic Index	593-599
151.	39-1	Antigenic Index	603-619
152.	39-1	Antigenic Index	626-628
153.	39-1	Antigenic Index	634-637
154.	39-1	Antigenic Index	639-647
155.	39-1	Antigenic Index	655-658
156.	39-1	Antigenic Index	672-674
157.	39-1	Antigenic Index	677-686
158.	39-1	Antigenic Index	688-691
159.	39-1	Antigenic Index	693-699
160.	39-1	Antigenic Index	707-710
161.	39-1	Hydrophilicity	28-32
162.	39-1	Hydrophilicity	38-44
163.	39-1	Hydrophilicity	54-69
164.	39-1	Hydrophilicity	80-83
165.	39-1	Hydrophilicity	89-96
166.	39-1	Hydrophilicity	117-119
167.	39-1	Hydrophilicity	121-123
168.	39-1	Hydrophilicity	152-154
169.	39-1	Hydrophilicity	224-231
170.	39-1	Hydrophilicity	247-265
171.	39-1	Hydrophilicity	318-332
172.	39-1	Hydrophilicity	357-361
173.	39-1	Hydrophilicity	402-404

174.	39-1	Hydrophilicity	406-408
175.	39-1	Hydrophilicity	446-449
176.	39-1	Hydrophilicity	454-459
177.	39-1	Hydrophilicity	465-469
178.	39-1	Hydrophilicity	476-487
179.	39-1	Hydrophilicity	491-499
180.	39-1	Hydrophilicity	506-514
181.	39-1	Hydrophilicity	525-535
182.	39-1	Hydrophilicity	560-567
183.	39-1	Hydrophilicity	573-575
184.	39-1	Hydrophilicity	577-580
185.	39-1	Hydrophilicity	594-596
186.	39-1	Hydrophilicity	605-607
187.	39-1	Hydrophilicity	611-619
188.	39-1	Hydrophilicity	634-637
189.	39-1	Hydrophilicity	639-647
190.	39-1	Hydrophilicity	672-674
191.	39-1	Hydrophilicity	677-686
192.	39-1	Hydrophilicity	688-690
193.	39-1	Hydrophilicity	693-695
194.	39a	AMPHI	6-13
195.	39a	AMPHI	21-24
196.	39a	AMPHI	37-40
197.	39a	AMPHI	60-75
198.	39a	AMPHI	118-122
199.	39a	AMPHI	134-139
200.	39a	AMPHI	165-183
201.	39a	AMPHI	192-195
202.	39a	AMPHI	233-241
203.	39a	AMPHI	247-267
204.	39a	AMPHI	273-275
205.	39a	AMPHI	299-308
206.	39a	AMPHI	310-319
207.	39a	AMPHI	322-330
208.	39a	AMPHI	338-347
209.	39a	AMPHI	358-364
210.	39a	AMPHI	366-368
211.	39a	AMPHI	376-378
212.	39a	AMPHI	385-392
213.	39a	AMPHI	413-416
214.	39a	AMPHI	421-424
215.	39a	AMPHI	429-438
216.	39a	AMPHI	445-454
217.	39a	AMPHI	456-458
218.	39a	AMPHI	498-500

219.	39a	AMPHI	512-520
220.	39a	AMPHI	576-587
221.	39a	AMPHI	589-600
222.	39a	AMPHI	650-652
223.	39a	AMPHI	670-674
224.	39a	Antigenic Index	26-32
225.	39a	Antigenic Index	35-45
226.	39a	Antigenic Index	54-69
227.	39a	Antigenic Index	79-84
228.	39a	Antigenic Index	89-96
229.	39a	Antigenic Index	103-110
230.	39a	Antigenic Index	117-124
231.	39a	Antigenic Index	152-154
232.	39a	Antigenic Index	190-192
233.	39a	Antigenic Index	222-231
234.	39a	Antigenic Index	246-265
235.	39a	Antigenic Index	292-295
236.	39a	Antigenic Index	318-335
237.	39a	Antigenic Index	353-362
238.	39a	Antigenic Index	370-372
239.	39a	Antigenic Index	402-404
240.	39a	Antigenic Index	406-408
241.	39a	Antigenic Index	419-421
242.	39a	Antigenic Index	446-449
243.	39a	Antigenic Index	453-460
244.	39a	Antigenic Index	465-469
245.	39a	Antigenic Index	476-487
246.	39a	Antigenic Index	491-499
247.	39a	Antigenic Index	505-514
248.	39a	Antigenic Index	529-535
249.	39a	Antigenic Index	557-567
250.	39a	Antigenic Index	569-575
251.	39a	Antigenic Index	577-580
252.	39a	Antigenic Index	593-599
253.	39a	Antigenic Index	603-619
254.	39a	Antigenic Index	626-628
255.	39a	Antigenic Index	634-637
256.	39a	Antigenic Index	639-647
257.	39a	Antigenic Index	655-658
258.	39a	Antigenic Index	672-674
259.	39a	Antigenic Index	677-686
260.	39a	Antigenic Index	688-691
261.	39a	Antigenic Index	693-699
262.	39a	Antigenic Index	707-710
263.	39a	Hydrophilicity	28-32

264.	39a	Hydrophilicity	38-44
265.	39a	Hydrophilicity	54-69
266.	39a	Hydrophilicity	80-83
267.	39a	Hydrophilicity	89-95
268.	39a	Hydrophilicity	105-108
269.	39a	Hydrophilicity	117-119
270.	39a	Hydrophilicity	121-123
271.	39a	Hydrophilicity	152-154
272.	39a	Hydrophilicity	224-231
273.	39a	Hydrophilicity	247-265
274.	39a	Hydrophilicity	318-332
275.	39a	Hydrophilicity	357-361
276.	39a	Hydrophilicity	402-404
277.	39a	Hydrophilicity	406-408
278.	39a	Hydrophilicity	446-449
279.	39a	Hydrophilicity	454-459
280.	39a	Hydrophilicity	465-469
281.	39a	Hydrophilicity	476-487
282.	39a	Hydrophilicity	491-499
283.	39a	Hydrophilicity	506-514
284.	39a	Hydrophilicity	529-535
285.	39a	Hydrophilicity	560-567
286.	39a	Hydrophilicity	573-575
287.	39a	Hydrophilicity	577-580
288.	39a	Hydrophilicity	594-596
289.	39a	Hydrophilicity	605-607
290.	39a	Hydrophilicity	611-619
291.	39a	Hydrophilicity	634-637
292.	39a	Hydrophilicity	639-647
293.	39a	Hydrophilicity	672-674
294.	39a	Hydrophilicity	677-686
295.	39a	Hydrophilicity	688-690
296.	39a	Hydrophilicity	693-695
297.	40-1	AMPHI	6-14
298.	40-1	AMPHI	16-19
299.	40-1	AMPHI	22-27
300.	40-1	AMPHI	30-33
301.	40-1	AMPHI	41-44
302.	40-1	AMPHI	62-68
303.	40-1	AMPHI	129-139
304.	40-1	AMPHI	161-165
305.	40-1	AMPHI	181-191
306.	40-1	AMPHI	199-202
307.	40-1	AMPHI	215-220
308.	40-1	AMPHI	237-249

309.	40-1	AMPHI	298-302
310.	40-1	AMPHI	313-318
311.	40-1	AMPHI	335-342
312.	40-1	AMPHI	376-383
313.	40-1	AMPHI	399-402
314.	40-1	AMPHI	426-428
315.	40-1	AMPHI	430-433
316.	40-1	AMPHI	435-437
317.	40-1	AMPHI	479-482
318.	40-1	AMPHI	491-511
319.	40-1	AMPHI	523-525
320.	40-1	AMPHI	560-563
321.	40-1	Antigenic Index	21-32
322.	40-1	Antigenic Index	49-61
323.	40-1	Antigenic Index	64-66
324.	40-1	Antigenic Index	74-92
325.	40-1	Antigenic Index	98-123
326.	40-1	Antigenic Index	129-135
327.	40-1	Antigenic Index	138-176
328.	40-1	Antigenic Index	193-195
329.	40-1	Antigenic Index	199-219
330.	40-1	Antigenic Index	226-240
331.	40-1	Antigenic Index	242-245
332.	40-1	Antigenic Index	251-257
333.	40-1	Antigenic Index	261-276
334.	40-1	Antigenic Index	279-306
335.	40-1	Antigenic Index	308-346
336.	40-1	Antigenic Index	352-367
337.	40-1	Antigenic Index	375-378
338.	40-1	Antigenic Index	384-406
339.	40-1	Antigenic Index	408-420
340.	40-1	Antigenic Index	423-426
341.	40-1	Antigenic Index	428-438
342.	40-1	Antigenic Index	453-459
343.	40-1	Antigenic Index	462-481
344.	40-1	Antigenic Index	485-494
345.	40-1	Antigenic Index	506-518
346.	40-1	Antigenic Index	535-539
347.	40-1	Antigenic Index	544-552
348.	40-1	Antigenic Index	559-566
349.	40-1	Antigenic Index	571-582
350.	40-1	Hydrophilicity	21-32
351.	40-1	Hydrophilicity	51-61
352.	40-1	Hydrophilicity	64-66
353.	40-1	Hydrophilicity	75-92

354.	40-1	Hydrophilicity	100-122
355.	40-1	Hydrophilicity	129-135
356.	40-1	Hydrophilicity	140-145
357.	40-1	Hydrophilicity	149-152
358.	40-1	Hydrophilicity	157-161
359.	40-1	Hydrophilicity	163-175
360.	40-1	Hydrophilicity	199-201
361.	40-1	Hydrophilicity	203-219
362.	40-1	Hydrophilicity	227-240
363.	40-1	Hydrophilicity	251-257
364.	40-1	Hydrophilicity	261-276
365.	40-1	Hydrophilicity	279-306
366.	40-1	Hydrophilicity	308-318
367.	40-1	Hydrophilicity	320-328
368.	40-1	Hydrophilicity	334-341
369.	40-1	Hydrophilicity	354-356
370.	40-1	Hydrophilicity	359-366
371.	40-1	Hydrophilicity	392-398
372.	40-1	Hydrophilicity	400-405
373.	40-1	Hydrophilicity	410-420
374.	40-1	Hydrophilicity	429-438
375.	40-1	Hydrophilicity	463-467
376.	40-1	Hydrophilicity	471-480
377.	40-1	Hydrophilicity	487-493
378.	40-1	Hydrophilicity	506-518
379.	40-1	Hydrophilicity	547-552
380.	40-1	Hydrophilicity	575-579
381.	40a	AMPHI	6-10
382.	40a	AMPHI	19-27
383.	40a	AMPHI	30-33
384.	40a	AMPHI	41-44
385.	40a	AMPHI	61-72
386.	40a	AMPHI	78-81
387.	40a	AMPHI	92-94
388.	40a	AMPHI	128-130
389.	40a	AMPHI	132-134
390.	40a	AMPHI	161-165
391.	40a	AMPHI	181-193
392.	40a	AMPHI	197-199
393.	40a	AMPHI	204-211
394.	40a	AMPHI	213-218
395.	40a	AMPHI	227-229
396.	40a	AMPHI	237-249
397.	40a	AMPHI	298-302
398.	40a	AMPHI	313-318

399.	40a	AMPHI	335-342
400.	40a	AMPHI	376-383
401.	40a	AMPHI	399-402
402.	40a	AMPHI	426-428
403.	40a	AMPHI	435-437
404.	40a	AMPHI	475-483
405.	40a	AMPHI	492-512
406.	40a	AMPHI	524-526
407.	40a	AMPHI	561-564
408.	40a	Antigenic Index	21-34
409.	40a	Antigenic Index	50-64
410.	40a	Antigenic Index	75-83
411.	40a	Antigenic Index	88-97
412.	40a	Antigenic Index	105-122
413.	40a	Antigenic Index	129-134
414.	40a	Antigenic Index	140-176
415.	40a	Antigenic Index	190-207
416.	40a	Antigenic Index	211-217
417.	40a	Antigenic Index	224-240
418.	40a	Antigenic Index	242-245
419.	40a	Antigenic Index	250-255
420.	40a	Antigenic Index	260-276
421.	40a	Antigenic Index	279-306
422.	40a	Antigenic Index	308-346
423.	40a	Antigenic Index	352-367
424.	40a	Antigenic Index	375-378
425.	40a	Antigenic Index	384-406
426.	40a	Antigenic Index	408-420
427.	40a	Antigenic Index	423-438
428.	40a	Antigenic Index	453-468
429.	40a	Antigenic Index	471-481
430.	40a	Antigenic Index	487-493
431.	40a	Antigenic Index	507-519
432.	40a	Antigenic Index	536-540
433.	40a	Antigenic Index	545-553
434.	40a	Antigenic Index	560-567
435.	40a	Antigenic Index	572-583
436.	40a	Hydrophilicity	21-34
437.	40a	Hydrophilicity	50-64
438.	40a	Hydrophilicity	75-83
439.	40a	Hydrophilicity	88-96
440.	40a	Hydrophilicity	105-121
441.	40a	Hydrophilicity	129-134
442.	40a	Hydrophilicity	140-145
443.	40a	Hydrophilicity	148-155

444.	40a	Hydrophilicity	157-161
445.	40a	Hydrophilicity	163-175
446.	40a	Hydrophilicity	196-202
447.	40a	Hydrophilicity	211-217
448.	40a	Hydrophilicity	225-230
449.	40a	Hydrophilicity	232-240
450.	40a	Hydrophilicity	253-255
451.	40a	Hydrophilicity	261-276
452.	40a	Hydrophilicity	279-306
453.	40a	Hydrophilicity	308-318
454.	40a	Hydrophilicity	320-328
455.	40a	Hydrophilicity	334-341
456.	40a	Hydrophilicity	354-356
457.	40a	Hydrophilicity	359-366
458.	40a	Hydrophilicity	392-398
459.	40a	Hydrophilicity	400-405
460.	40a	Hydrophilicity	410-420
461.	40a	Hydrophilicity	428-438
462.	40a	Hydrophilicity	462-468
463.	40a	Hydrophilicity	472-481
464.	40a	Hydrophilicity	489-493
465.	40a	Hydrophilicity	507-519
466.	40a	Hydrophilicity	548-553
467.	40a	Hydrophilicity	576-580
468.	41-1	AMPHI	30-36
469.	41-1	AMPHI	93-98
470.	41-1	AMPHI	111-122
471.	41-1	AMPHI	126-129
472.	41-1	AMPHI	136-143
473.	41-1	AMPHI	145-150
474.	41-1	AMPHI	156-158
475.	41-1	AMPHI	186-195
476.	41-1	AMPHI	201-208
477.	41-1	AMPHI	213-223
478.	41-1	AMPHI	236-247
479.	41-1	AMPHI	250-255
480.	41-1	AMPHI	273-282
481.	41-1	AMPHI	303-309
482.	41-1	AMPHI	311-314
483.	41-1	AMPHI	329-338
484.	41-1	AMPHI	344-362
485.	41-1	AMPHI	372-377
486.	41-1	AMPHI	385-392
487.	41-1	AMPHI	409-412
488.	41-1	AMPHI	419-426

489.	41-1	AMPHI	458-463
490.	41-1	AMPHI	470-474
491.	41-1	AMPHI	486-489
492.	41-1	AMPHI	512-518
493.	41-1	AMPHI	527-551
494.	41-1	AMPHI	564-579
495.	41-1	AMPHI	593-597
496.	41-1	Antigenic Index	13-22
497.	41-1	Antigenic Index	30-38
498.	41-1	Antigenic Index	43-55
499.	41-1	Antigenic Index	73-75
500.	41-1	Antigenic Index	87-89
501.	41-1	Antigenic Index	105-112
502.	41-1	Antigenic Index	114-124
503.	41-1	Antigenic Index	136-141
504.	41-1	Antigenic Index	147-153
505.	41-1	Antigenic Index	163-166
506.	41-1	Antigenic Index	174-184
507.	41-1	Antigenic Index	195-207
508.	41-1	Antigenic Index	226-236
509.	41-1	Antigenic Index	244-246
510.	41-1	Antigenic Index	249-265
511.	41-1	Antigenic Index	281-287
512.	41-1	Antigenic Index	294-313
513.	41-1	Antigenic Index	317-342
514.	41-1	Antigenic Index	350-375
515.	41-1	Antigenic Index	379-386
516.	41-1	Antigenic Index	390-396
517.	41-1	Antigenic Index	413-422
518.	41-1	Antigenic Index	425-430
519.	41-1	Antigenic Index	436-440
520.	41-1	Antigenic Index	446-465
521.	41-1	Antigenic Index	468-495
522.	41-1	Antigenic Index	498-518
523.	41-1	Antigenic Index	520-522
524.	41-1	Antigenic Index	525-542
525.	41-1	Antigenic Index	547-558
526.	41-1	Antigenic Index	565-590
527.	41-1	Antigenic Index	595-602
528.	41-1	Antigenic Index	608-619
529.	41-1	Hydrophilicity	14-21
530.	41-1	Hydrophilicity	30-33
531.	41-1	Hydrophilicity	45-55
532.	41-1	Hydrophilicity	87-89
533.	41-1	Hydrophilicity	106-111

534.	41-1	Hydrophilicity	114-120
535.	41-1	Hydrophilicity	122-124
536.	41-1	Hydrophilicity	136-141
537.	41-1	Hydrophilicity	148-150
538.	41-1	Hydrophilicity	177-184
539.	41-1	Hydrophilicity	195-207
540.	41-1	Hydrophilicity	226-234
541.	41-1	Hydrophilicity	249-265
542.	41-1	Hydrophilicity	285-287
543.	41-1	Hydrophilicity	294-297
544.	41-1	Hydrophilicity	299-313
545.	41-1	Hydrophilicity	317-321
546.	41-1	Hydrophilicity	323-342
547.	41-1	Hydrophilicity	350-371
548.	41-1	Hydrophilicity	379-386
549.	41-1	Hydrophilicity	417-422
550.	41-1	Hydrophilicity	425-427
551.	41-1	Hydrophilicity	447-449
552.	41-1	Hydrophilicity	459-462
553.	41-1	Hydrophilicity	468-475
554.	41-1	Hydrophilicity	479-482
555.	41-1	Hydrophilicity	484-491
556.	41-1	Hydrophilicity	499-518
557.	41-1	Hydrophilicity	520-522
558.	41-1	Hydrophilicity	526-542
559.	41-1	Hydrophilicity	550-558
560.	41-1	Hydrophilicity	568-590
561.	41-1	Hydrophilicity	595-598
562.	41-1	Hydrophilicity	617-619
563.	41a	AMPHI	6-12
564.	41a	AMPHI	32-34
565.	41a	AMPHI	69-74
566.	41a	AMPHI	86-98
567.	41a	AMPHI	111-119
568.	41a	AMPHI	121-126
569.	41a	AMPHI	132-134
570.	41a	AMPHI	155-160
571.	41a	AMPHI	162-171
572.	41a	AMPHI	177-184
573.	41a	AMPHI	189-199
574.	41a	AMPHI	212-223
575.	41a	AMPHI	226-231
576.	41a	AMPHI	249-258
577.	41a	AMPHI	287-290
578.	41a	AMPHI	305-314

579.	41a	AMPHI	320-338
580.	41a	AMPHI	348-353
581.	41a	AMPHI	361-368
582.	41a	AMPHI	385-388
583.	41a	AMPHI	395-402
584.	41a	AMPHI	434-439
585.	41a	AMPHI	446-450
586.	41a	AMPHI	462-467
587.	41a	AMPHI	470-475
588.	41a	AMPHI	488-494
589.	41a	AMPHI	503-525
590.	41a	AMPHI	540-555
591.	41a	AMPHI	569-573
592.	41a	AMPHI	578-594
593.	41a	Antigenic Index	10-13
594.	41a	Antigenic Index	19-31
595.	41a	Antigenic Index	48-50
596.	41a	Antigenic Index	63-65
597.	41a	Antigenic Index	82-101
598.	41a	Antigenic Index	112-117
599.	41a	Antigenic Index	123-129
600.	41a	Antigenic Index	139-142
601.	41a	Antigenic Index	150-160
602.	41a	Antigenic Index	171-183
603.	41a	Antigenic Index	202-212
604.	41a	Antigenic Index	220-222
605.	41a	Antigenic Index	225-241
606.	41a	Antigenic Index	257-263
607.	41a	Antigenic Index	270-289
608.	41a	Antigenic Index	293-318
609.	41a	Antigenic Index	326-351
610.	41a	Antigenic Index	355-362
611.	41a	Antigenic Index	366-372
612.	41a	Antigenic Index	389-398
613.	41a	Antigenic Index	401-406
614.	41a	Antigenic Index	412-416
615.	41a	Antigenic Index	422-441
616.	41a	Antigenic Index	444-446
617.	41a	Antigenic Index	451-471
618.	41a	Antigenic Index	475-494
619.	41a	Antigenic Index	496-498
620.	41a	Antigenic Index	501-518
621.	41a	Antigenic Index	523-534
622.	41a	Antigenic Index	540-566
623.	41a	Antigenic Index	571-578

624.	41a	Antigenic Index	582-595
625.	41a	Hydrophilicity	21-31
626.	41a	Hydrophilicity	63-65
627.	41a	Hydrophilicity	83-96
628.	41a	Hydrophilicity	98-100
629.	41a	Hydrophilicity	112-117
630.	41a	Hydrophilicity	124-126
631.	41a	Hydrophilicity	153-160
632.	41a	Hydrophilicity	171-183
633.	41a	Hydrophilicity	202-210
634.	41a	Hydrophilicity	220-222
635.	41a	Hydrophilicity	225-241
636.	41a	Hydrophilicity	261-263
637.	41a	Hydrophilicity	270-273
638.	41a	Hydrophilicity	275-289
639.	41a	Hydrophilicity	293-297
640.	41a	Hydrophilicity	299-318
641.	41a	Hydrophilicity	326-347
642.	41a	Hydrophilicity	355-362
643.	41a	Hydrophilicity	393-398
644.	41a	Hydrophilicity	401-403
645.	41a	Hydrophilicity	423-425
646.	41a	Hydrophilicity	435-438
647.	41a	Hydrophilicity	454-458
648.	41a	Hydrophilicity	460-471
649.	41a	Hydrophilicity	475-494
650.	41a	Hydrophilicity	496-498
651.	41a	Hydrophilicity	502-518
652.	41a	Hydrophilicity	527-534
653.	41a	Hydrophilicity	544-566
654.	41a	Hydrophilicity	571-574
655.	41a	Hydrophilicity	593-595
656.	44-1	AMPHI	57-60
657.	44-1	AMPHI	76-79
658.	44-1	Antigenic Index	22-34
659.	44-1	Antigenic Index	38-46
660.	44-1	Antigenic Index	50-55
661.	44-1	Antigenic Index	64-70
662.	44-1	Antigenic Index	72-80
663.	44-1	Antigenic Index	83-89
664.	44-1	Antigenic Index	96-106
665.	44-1	Antigenic Index	110-124
666.	44-1	Hydrophilicity	22-34
667.	44-1	Hydrophilicity	40-46
668.	44-1	Hydrophilicity	64-69

669.	44-1	Hydrophilicity	73-80
670.	44-1	Hydrophilicity	84-89
671.	44-1	Hydrophilicity	97-106
672.	44-1	Hydrophilicity	120-124
673.	44a	AMPHI	57-60
674.	44a	AMPHI	76-79
675.	44a	Antigenic Index	23-34
676.	44a	Antigenic Index	38-46
677.	44a	Antigenic Index	50-55
678.	44a	Antigenic Index	64-70
679.	44a	Antigenic Index	72-80
680.	44a	Antigenic Index	83-89
681.	44a	Antigenic Index	96-106
682.	44a	Antigenic Index	110-124
683.	44a	Hydrophilicity	28-34
684.	44a	Hydrophilicity	40-46
685.	44a	Hydrophilicity	64-69
686.	44a	Hydrophilicity	73-80
687.	44a	Hydrophilicity	84-89
688.	44a	Hydrophilicity	97-106
689.	44a	Hydrophilicity	120-124
690.	49-1	AMPHI	16-21
691.	49-1	AMPHI	44-48
692.	49-1	AMPHI	56-61
693.	49-1	AMPHI	92-97
694.	49-1	AMPHI	118-127
695.	49-1	AMPHI	130-149
696.	49-1	AMPHI	156-178
697.	49-1	AMPHI	235-240
698.	49-1	AMPHI	253-264
699.	49-1	AMPHI	268-271
700.	49-1	AMPHI	278-285
701.	49-1	AMPHI	287-292
702.	49-1	AMPHI	298-300
703.	49-1	AMPHI	328-337
704.	49-1	AMPHI	343-350
705.	49-1	AMPHI	355-365
706.	49-1	AMPHI	378-389
707.	49-1	AMPHI	422-424
708.	49-1	AMPHI	442-450
709.	49-1	AMPHI	464-481
710.	49-1	AMPHI	486-496
711.	49-1	AMPHI	514-521
712.	49-1	AMPHI	548-551
713.	49-1	AMPHI	553-557

714.	49-1	AMPHI	562-568
715.	49-1	AMPHI	573-575
716.	49-1	AMPHI	588-590
717.	49-1	AMPHI	603-605
718.	49-1	AMPHI	614-618
719.	49-1	Antigenic Index	15-21
720.	49-1	Antigenic Index	26-43
721.	49-1	Antigenic Index	50-59
722.	49-1	Antigenic Index	61-75
723.	49-1	Antigenic Index	79-87
724.	49-1	Antigenic Index	98-108
725.	49-1	Antigenic Index	110-120
726.	49-1	Antigenic Index	122-139
727.	49-1	Antigenic Index	147-164
728.	49-1	Antigenic Index	171-179
729.	49-1	Antigenic Index	185-197
730.	49-1	Antigenic Index	214-216
731.	49-1	Antigenic Index	229-231
732.	49-1	Antigenic Index	248-266
733.	49-1	Antigenic Index	278-283
734.	49-1	Antigenic Index	289-295
735.	49-1	Antigenic Index	316-326
736.	49-1	Antigenic Index	337-349
737.	49-1	Antigenic Index	368-378
738.	49-1	Antigenic Index	386-388
739.	49-1	Antigenic Index	390-410
740.	49-1	Antigenic Index	412-414
741.	49-1	Antigenic Index	423-429
742.	49-1	Antigenic Index	438-454
743.	49-1	Antigenic Index	462-475
744.	49-1	Antigenic Index	482-500
745.	49-1	Antigenic Index	503-509
746.	49-1	Antigenic Index	521-528
747.	49-1	Antigenic Index	540-562
748.	49-1	Antigenic Index	572-579
749.	49-1	Antigenic Index	590-606
750.	49-1	Antigenic Index	610-612
751.	49-1	Antigenic Index	617-619
752.	49-1	Antigenic Index	626-634
753.	49-1	Antigenic Index	637-640
754.	49-1	Hydrophilicity	18-21
755.	49-1	Hydrophilicity	26-29
756.	49-1	Hydrophilicity	31-43
757.	49-1	Hydrophilicity	51-57
758.	49-1	Hydrophilicity	64-68

759.	49-1	Hydrophilicity	79-87
760.	49-1	Hydrophilicity	98-107
761.	49-1	Hydrophilicity	122-125
762.	49-1	Hydrophilicity	147-164
763.	49-1	Hydrophilicity	172-175
764.	49-1	Hydrophilicity	187-197
765.	49-1	Hydrophilicity	229-231
766.	49-1	Hydrophilicity	256-262
767.	49-1	Hydrophilicity	264-266
768.	49-1	Hydrophilicity	278-283
769.	49-1	Hydrophilicity	290-292
770.	49-1	Hydrophilicity	319-326
771.	49-1	Hydrophilicity	337-349
772.	49-1	Hydrophilicity	368-376
773.	49-1	Hydrophilicity	386-388
774.	49-1	Hydrophilicity	390-410
775.	49-1	Hydrophilicity	412-414
776.	49-1	Hydrophilicity	423-429
777.	49-1	Hydrophilicity	441-451
778.	49-1	Hydrophilicity	466-472
779.	49-1	Hydrophilicity	484-490
780.	49-1	Hydrophilicity	492-494
781.	49-1	Hydrophilicity	496-498
782.	49-1	Hydrophilicity	522-528
783.	49-1	Hydrophilicity	543-562
784.	49-1	Hydrophilicity	591-606
785.	49-1	Hydrophilicity	617-619
786.	49-1	Hydrophilicity	626-632
787.	49-1	Hydrophilicity	637-640
788.	49a	AMPHI	55-61
789.	49a	AMPHI	92-97
790.	49a	AMPHI	118-127
791.	49a	AMPHI	129-135
792.	49a	AMPHI	137-145
793.	49a	AMPHI	156-178
794.	49a	AMPHI	198-200
795.	49a	AMPHI	235-240
796.	49a	AMPHI	252-264
797.	49a	AMPHI	277-285
798.	49a	AMPHI	287-292
799.	49a	AMPHI	298-300
800.	49a	AMPHI	321-326
801.	49a	AMPHI	328-337
802.	49a	AMPHI	343-350
803.	49a	AMPHI	355-365

804.	49a	AMPHI	378-389
805.	49a	AMPHI	392-397
806.	49a	AMPHI	415-424
807.	49a	AMPHI	453-456
808.	49a	AMPHI	471-480
809.	49a	AMPHI	486-504
810.	49a	AMPHI	514-519
811.	49a	AMPHI	527-534
812.	49a	AMPHI	551-554
813.	49a	AMPHI	561-568
814.	49a	AMPHI	600-605
815.	49a	AMPHI	612-616
816.	49a	AMPHI	628-633
817.	49a	AMPHI	636-641
818.	49a	AMPHI	654-660
819.	49a	AMPHI	669-691
820.	49a	AMPHI	706-721
821.	49a	AMPHI	735-739
822.	49a	AMPHI	744-760
823.	49a	Antigenic Index	4-23
824.	49a	Antigenic Index	27-43
825.	49a	Antigenic Index	51-62
826.	49a	Antigenic Index	64-68
827.	49a	Antigenic Index	72-75
828.	49a	Antigenic Index	79-87
829.	49a	Antigenic Index	98-108
830.	49a	Antigenic Index	110-120
831.	49a	Antigenic Index	124-139
832.	49a	Antigenic Index	147-164
833.	49a	Antigenic Index	176-179
834.	49a	Antigenic Index	185-197
835.	49a	Antigenic Index	214-216
836.	49a	Antigenic Index	229-231
837.	49a	Antigenic Index	248-267
838.	49a	Antigenic Index	278-283
839.	49a	Antigenic Index	289-295
840.	49a	Antigenic Index	305-308
841.	49a	Antigenic Index	316-326
842.	49a	Antigenic Index	337-349
843.	49a	Antigenic Index	368-378
844.	49a	Antigenic Index	386-388
845.	49a	Antigenic Index	391-407
846.	49a	Antigenic Index	423-429
847.	49a	Antigenic Index	436-455
848.	49a	Antigenic Index	459-484

849.	49a	Antigenic Index	492-517
850.	49a	Antigenic Index	521-528
851.	49a	Antigenic Index	532-539
852.	49a	Antigenic Index	555-564
853.	49a	Antigenic Index	567-572
854.	49a	Antigenic Index	578-582
855.	49a	Antigenic Index	588-607
856.	49a	Antigenic Index	610-612
857.	49a	Antigenic Index	617-637
858.	49a	Antigenic Index	641-660
859.	49a	Antigenic Index	662-664
860.	49a	Antigenic Index	667-684
861.	49a	Antigenic Index	689-700
862.	49a	Antigenic Index	706-732
863.	49a	Antigenic Index	737-744
864.	49a	Antigenic Index	748-761
865.	49a	Hydrophilicity	4-23
866.	49a	Hydrophilicity	31-43
867.	49a	Hydrophilicity	51-53
868.	49a	Hydrophilicity	55-57
869.	49a	Hydrophilicity	64-68
870.	49a	Hydrophilicity	79-87
871.	49a	Hydrophilicity	98-106
872.	49a	Hydrophilicity	114-120
873.	49a	Hydrophilicity	130-139
874.	49a	Hydrophilicity	147-164
875.	49a	Hydrophilicity	187-197
876.	49a	Hydrophilicity	229-231
877.	49a	Hydrophilicity	249-262
878.	49a	Hydrophilicity	264-266
879.	49a	Hydrophilicity	278-283
880.	49a	Hydrophilicity	290-292
881.	49a	Hydrophilicity	319-326
882.	49a	Hydrophilicity	337-349
883.	49a	Hydrophilicity	368-376
884.	49a	Hydrophilicity	386-388
885.	49a	Hydrophilicity	391-407
886.	49a	Hydrophilicity	427-429
887.	49a	Hydrophilicity	436-439
888.	49a	Hydrophilicity	441-455
889.	49a	Hydrophilicity	459-463
890.	49a	Hydrophilicity	465-484
891.	49a	Hydrophilicity	492-513
892.	49a	Hydrophilicity	521-528
893.	49a	Hydrophilicity	559-564

894.	49a	Hydrophilicity	567-569
895.	49a	Hydrophilicity	589-591
896.	49a	Hydrophilicity	601-604
897.	49a	Hydrophilicity	620-624
898.	49a	Hydrophilicity	626-637
899.	49a	Hydrophilicity	641-660
900.	49a	Hydrophilicity	662-664
901.	49a	Hydrophilicity	668-684
902.	49a	Hydrophilicity	693-700
903.	49a	Hydrophilicity	710-732
904.	49a	Hydrophilicity	737-740
905.	49a	Hydrophilicity	759-761
906.	51-1	AMPHI	15-21
907.	51-1	AMPHI	40-54
908.	51-1	AMPHI	75-86
909.	51-1	AMPHI	108-110
910.	51-1	AMPHI	112-124
911.	51-1	AMPHI	141-148
912.	51-1	AMPHI	184-189
913.	51-1	AMPHI	211-216
914.	51-1	Antigenic Index	58-65
915.	51-1	Antigenic Index	123-127
916.	51-1	Antigenic Index	132-137
917.	51-1	Antigenic Index	149-153
918.	51-1	Antigenic Index	165-177
919.	51-1	Antigenic Index	198-204
920.	51-1	Antigenic Index	222-231
921.	51-1	Hydrophilicity	60-65
922.	51-1	Hydrophilicity	123-127
923.	51-1	Hydrophilicity	132-135
924.	51-1	Hydrophilicity	165-174
925.	51-1	Hydrophilicity	200-203
926.	51-1	Hydrophilicity	222-227
927.	51a	AMPHI	15-21
928.	51a	AMPHI	40-54
929.	51a	AMPHI	75-86
930.	51a	AMPHI	108-110
931.	51a	AMPHI	112-124
932.	51a	AMPHI	141-148
933.	51a	AMPHI	184-189
934.	51a	AMPHI	211-216
935.	51a	Hydrophilicity	60-65
936.	51a	Hydrophilicity	123-127
937.	51a	Hydrophilicity	132-135
938.	51a	Hydrophilicity	165-174

939.	51a	Hydrophilicity	200-203
940.	51a	Hydrophilicity	222-227
941.	52-1	AMPHI	48-50
942.	52-1	AMPHI	64-73
943.	52-1	Antigenic Index	19-26
944.	52-1	Antigenic Index	30-35
945.	52-1	Antigenic Index	42-52
946.	52-1	Antigenic Index	57-86
947.	52-1	Hydrophilicity	22-26
948.	52-1	Hydrophilicity	30-35
949.	52-1	Hydrophilicity	42-52
950.	52-1	Hydrophilicity	57-71
951.	52-1	Hydrophilicity	78-86
952.	69-1	AMPHI	25-27
953.	69-1	AMPHI	46-66
954.	69-1	Antigenic Index	32-41
955.	69-1	Antigenic Index	43-45
956.	69-1	Antigenic Index	71-78
957.	69-1	Hydrophilicity	32-38
958.	69-1	Hydrophilicity	71-78
959.	69a	AMPHI	25-27
960.	69a	AMPHI	46-66
961.	69a	Antigenic Index	32-41
962.	69a	Antigenic Index	43-46
963.	69a	Antigenic Index	71-78
964.	69a	Hydrophilicity	32-38
965.	69a	Hydrophilicity	71-78
966.	77-1	AMPHI	12-16
967.	77-1	AMPHI	23-33
968.	77-1	AMPHI	35-42
969.	77-1	AMPHI	51-57
970.	77-1	AMPHI	67-70
971.	77-1	AMPHI	73-79
972.	77-1	AMPHI	122-124
973.	77-1	AMPHI	130-134
974.	77-1	AMPHI	165-178
975.	77-1	AMPHI	191-211
976.	77-1	Antigenic Index	22-31
977.	77-1	Antigenic Index	34-44
978.	77-1	Antigenic Index	80-94
979.	77-1	Antigenic Index	101-104
980.	77-1	Antigenic Index	155-158
981.	77-1	Antigenic Index	167-181
982.	77-1	Hydrophilicity	22-28
983.	77-1	Hydrophilicity	38-44

984.	77-1	Hydrophilicity	80-92
985.	77-1	Hydrophilicity	171-178
986.	77a	AMPHI	8-15
987.	77a	AMPHI	24-30
988.	77a	AMPHI	40-43
989.	77a	AMPHI	46-52
990.	77a	AMPHI	95-97
991.	77a	AMPHI	103-107
992.	77a	AMPHI	114-125
993.	77a	AMPHI	144-151
994.	77a	AMPHI	154-156
995.	77a	AMPHI	166-184
996.	77a	Antigenic Index	7-17
997.	77a	Antigenic Index	53-67
998.	77a	Antigenic Index	74-77
999.	77a	Antigenic Index	128-131
1000.	77a	Antigenic Index	140-154
1001.	77a	Hydrophilicity	11-17
1002.	77a	Hydrophilicity	53-65
1003.	77a	Hydrophilicity	141-151
1004.	81-1	AMPHI	30-40
1005.	81-1	AMPHI	54-56
1006.	81-1	AMPHI	60-63
1007.	81-1	AMPHI	76-93
1008.	81-1	AMPHI	96-101
1009.	81-1	AMPHI	104-406
1010.	81-1	AMPHI	118-126
1011.	81-1	AMPHI	190-205
1012.	81-1	AMPHI	230-233
1013.	81-1	AMPHI	239-242
1014.	81-1	AMPHI	256-258
1015.	81-1	AMPHI	264-284
1016.	81-1	AMPHI	290-297
1017.	81-1	AMPHI	317-326
1018.	81-1	AMPHI	388-396
1019.	81-1	AMPHI	403-414
1020.	81-1	AMPHI	458-463
1021.	81-1	AMPHI	476-480
1022.	81-1	Antigenic Index	1-4
1023.	81-1	Antigenic Index	35-38
1024.	81-1	Antigenic Index	86-89
1025.	81-1	Antigenic Index	95-98
1026.	81-1	Antigenic Index	100-103
1027.	81-1	Antigenic Index	128-136
1028.	81-1	Antigenic Index	154-174

1029.	81-1	Antigenic Index	197-211
1030.	81-1	Antigenic Index	220-226
1031.	81-1	Antigenic Index	232-240
1032.	81-1	Antigenic Index	244-249
1033.	81-1	Antigenic Index	251-253
1034.	81-1	Antigenic Index	255-258
1035.	81-1	Antigenic Index	276-290
1036.	81-1	Antigenic Index	292-301
1037.	81-1	Antigenic Index	307-312
1038.	81-1	Antigenic Index	318-323
1039.	81-1	Antigenic Index	334-345
1040.	81-1	Antigenic Index	352-358
1041.	81-1	Antigenic Index	364-372
1042.	81-1	Antigenic Index	376-384
1043.	81-1	Antigenic Index	387-401
1044.	81-1	Antigenic Index	409-417
1045.	81-1	Antigenic Index	423-444
1046.	81-1	Antigenic Index	452-459
1047.	81-1	Antigenic Index	486-488
1048.	81-1	Antigenic Index	490-499
1049.	81-1	Antigenic Index	507-520
1050.	81-1	Hydrophilicity	1-4
1051.	81-1	Hydrophilicity	35-38
1052.	81-1	Hydrophilicity	95-98
1053.	81-1	Hydrophilicity	128-136
1054.	81-1	Hydrophilicity	154-164
1055.	81-1	Hydrophilicity	166-172
1056.	81-1	Hydrophilicity	202-209
1057.	81-1	Hydrophilicity	220-226
1058.	81-1	Hydrophilicity	234-238
1059.	81-1	Hydrophilicity	245-249
1060.	81-1	Hydrophilicity	251-253
1061.	81-1	Hydrophilicity	284-287
1062.	81-1	Hydrophilicity	292-299
1063.	81-1	Hydrophilicity	307-312
1064.	81-1	Hydrophilicity	321-323
1065.	81-1	Hydrophilicity	338-345
1066.	81-1	Hydrophilicity	366-368
1067.	81-1	Hydrophilicity	378-384
1068.	81-1	Hydrophilicity	387-401
1069.	81-1	Hydrophilicity	409-415
1070.	81-1	Hydrophilicity	453-459
1071.	81-1	Hydrophilicity	493-499
1072.	81-1	Hydrophilicity	507-509
1073.	81-1	Hydrophilicity	512-518

1074.	82a	AMPHI	36-40
1075.	82a	AMPHI	95-111
1076.	82a	AMPHI	117-132
1077.	82a	AMPHI	135-137
1078.	82a	AMPHI	160-174
1079.	82a	AMPHI	183-187
1080.	82a	Antigenic Index	2-8
1081.	82a	Antigenic Index	56-60
1082.	82a	Antigenic Index	90-97
1083.	82a	Antigenic Index	104-111
1084.	82a	Antigenic Index	114-137
1085.	82a	Antigenic Index	141-151
1086.	82a	Antigenic Index	170-175
1087.	82a	Antigenic Index	180-188
1088.	82a	Antigenic Index	194-201
1089.	82a	Antigenic Index	206-209
1090.	82a	Antigenic Index	216-218
1091.	82a	Hydrophilicity	2-8
1092.	82a	Hydrophilicity	56-60
1093.	82a	Hydrophilicity	90-97
1094.	82a	Hydrophilicity	105-108
1095.	82a	Hydrophilicity	120-128
1096.	82a	Hydrophilicity	130-134
1097.	82a	Hydrophilicity	141-151
1098.	82a	Hydrophilicity	170-175
1099.	82a	Hydrophilicity	186-188
1100.	82a	Hydrophilicity	195-201
1101.	82a	Hydrophilicity	206-209
1102.	112-1	AMPHI	6-8
1103.	112-1	AMPHI	12-34
1104.	112-1	AMPHI	45-53
1105.	112-1	AMPHI	63-65
1106.	112-1	AMPHI	70-82
1107.	112-1	AMPHI	84-86
1108.	112-1	AMPHI	107-109
1109.	112-1	AMPHI	116-123
1110.	112-1	AMPHI	183-186
1111.	112-1	AMPHI	244-246
1112.	112-1	AMPHI	248-258
1113.	112-1	AMPHI	280-282
1114.	112-1	AMPHI	302-313
1115.	112-1	Antigenic Index	35-44
1116.	112-1	Antigenic Index	57-61
1117.	112-1	Antigenic Index	81-84
1118.	112-1	Antigenic Index	91-98

1119.	112-1	Antigenic Index	125-133
1120.	112-1	Antigenic Index	140-147
1121.	112-1	Antigenic Index	149-159
1122.	112-1	Antigenic Index	161-165
1123.	112-1	Antigenic Index	174-190
1124.	112-1	Antigenic Index	192-200
1125.	112-1	Antigenic Index	202-216
1126.	112-1	Antigenic Index	218-224
1127.	112-1	Antigenic Index	228-232
1128.	112-1	Antigenic Index	239-244
1129.	112-1	Antigenic Index	255-263
1130.	112-1	Antigenic Index	290-300
1131.	112-1	Hydrophilicity	38-40
1132.	112-1	Hydrophilicity	57-61
1133.	112-1	Hydrophilicity	92-98
1134.	112-1	Hydrophilicity	125-133
1135.	112-1	Hydrophilicity	141-143
1136.	112-1	Hydrophilicity	150-159
1137.	112-1	Hydrophilicity	161-164
1138.	112-1	Hydrophilicity	175-190
1139.	112-1	Hydrophilicity	203-216
1140.	112-1	Hydrophilicity	218-224
1141.	112-1	Hydrophilicity	228-232
1142.	112-1	Hydrophilicity	239-244
1143.	112-1	Hydrophilicity	259-261
1144.	112-1	Hydrophilicity	293-297
1145.	112a	AMPHI	6-8
1146.	112a	AMPHI	12-34
1147.	112a	AMPHI	47-54
1148.	112a	AMPHI	63-65
1149.	112a	AMPHI	69-72
1150.	112a	AMPHI	84-86
1151.	112a	AMPHI	89-91
1152.	112a	AMPHI	107-109
1153.	112a	AMPHI	116-123
1154.	112a	AMPHI	183-186
1155.	112a	AMPHI	244-246
1156.	112a	AMPHI	248-258
1157.	112a	AMPHI	280-282
1158.	112a	AMPHI	302-310
1159.	112a	AMPHI	321-336
1160.	112a	Antigenic Index	35-44
1161.	112a	Antigenic Index	57-61
1162.	112a	Antigenic Index	81-84
1163.	112a	Antigenic Index	91-98

1164.	112a	Antigenic Index	125-133
1165.	112a	Antigenic Index	140-147
1166.	112a	Antigenic Index	150-158
1167.	112a	Antigenic Index	161-164
1168.	112a	Antigenic Index	174-190
1169.	112a	Antigenic Index	194-200
1170.	112a	Antigenic Index	202-216
1171.	112a	Antigenic Index	218-220
1172.	112a	Antigenic Index	222-224
1173.	112a	Antigenic Index	228-232
1174.	112a	Antigenic Index	239-244
1175.	112a	Antigenic Index	256-263
1176.	112a	Antigenic Index	290-301
1177.	112a	Antigenic Index	351-356
1178.	112a	Hydrophilicity	38-40
1179.	112a	Hydrophilicity	57-61
1180.	112a	Hydrophilicity	93-98
1181.	112a	Hydrophilicity	125-133
1182.	112a	Hydrophilicity	141-143
1183.	112a	Hydrophilicity	150-155
1184.	112a	Hydrophilicity	161-164
1185.	112a	Hydrophilicity	175-190
1186.	112a	Hydrophilicity	203-216
1187.	112a	Hydrophilicity	218-220
1188.	112a	Hydrophilicity	222-224
1189.	112a	Hydrophilicity	228-232
1190.	112a	Hydrophilicity	239-244
1191.	112a	Hydrophilicity	259-261
1192.	112a	Hydrophilicity	293-297
1193.	112a	Hydrophilicity	351-356
1194.	114-1	AMPHI	45-54
1195.	114-1	AMPHI	154-160
1196.	114-1	AMPHI	182-190
1197.	114-1	AMPHI	224-226
1198.	114-1	AMPHI	229-233
1199.	114-1	AMPHI	285-287
1200.	114-1	AMPHI	303-310
1201.	114-1	AMPHI	321-332
1202.	114-1	AMPHI	392-398
1203.	114-1	AMPHI	413-416
1204.	114-1	AMPHI	450-452
1205.	114-1	AMPHI	477-487
1206.	114-1	AMPHI	506-509
1207.	114-1	AMPHI	525-529
1208.	114-1	AMPHI	565-567

1209.	114-1	AMPHI	614-621
1210.	114-1	AMPHI	631-635
1211.	114-1	AMPHI	770-774
1212.	114-1	AMPHI	810-813
1213.	114-1	AMPHI	847-849
1214.	114-1	AMPHI	851-853
1215.	114-1	AMPHI	875-879
1216.	114-1	AMPHI	951-956
1217.	114-1	AMPHI	975-980
1218.	114-1	AMPHI	1034-1036
1219.	114-1	AMPHI	1048-1051
1220.	114-1	AMPHI	1073-1081
1221.	114-1	AMPHI	1086-1090
1222.	114-1	AMPHI	1095-1102
1223.	114-1	AMPHI	1111-1115
1224.	114-1	AMPHI	1163-1167
1225.	114-1	AMPHI	1242-1245
1226.	114-1	AMPHI	1275-1281
1227.	114-1	AMPHI	1312-1317
1228.	114-1	AMPHI	1338-1347
1229.	114-1	AMPHI	1349-1355
1230.	114-1	AMPHI	1357-1360
1231.	114-1	AMPHI	1362-1365
1232.	114-1	AMPHI	1376-1398
1233.	114-1	AMPHI	1418-1421
1234.	114-1	AMPHI	1425-1429
1235.	114-1	AMPHI	1468-1473
1236.	114-1	AMPHI	1476-1485
1237.	114-1	AMPHI	1495-1515
1238.	114-1	AMPHI	1518-1526
1239.	114-1	AMPHI	1546-1555
1240.	114-1	AMPHI	1557-1559
1241.	114-1	AMPHI	1580-1583
1242.	114-1	AMPHI	1585-1597
1243.	114-1	AMPHI	1604-1606
1244.	114-1	AMPHI	1613-1624
1245.	114-1	AMPHI	1626-1630
1246.	114-1	AMPHI	1638-1644
1247.	114-1	AMPHI	1655-1660
1248.	114-1	AMPHI	1662-1664
1249.	114-1	AMPHI	1672-1674
1250.	114-1	AMPHI	1677-1679
1251.	114-1	AMPHI	1691-1694
1252.	114-1	AMPHI	1713-1716
1253.	114-1	AMPHI	1719-1729

1254.	114-1	AMPHI	1735-1738
1255.	114-1	AMPHI	1753-1757
1256.	114-1	AMPHI	1772-1778
1257.	114-1	AMPHI	1790-1792
1258.	114-1	AMPHI	1817-1826
1259.	114-1	AMPHI	1828-1832
1260.	114-1	AMPHI	1840-1851
1261.	114-1	AMPHI	1854-1856
1262.	114-1	AMPHI	1871-1881
1263.	114-1	AMPHI	1883-1896
1264.	114-1	AMPHI	1922-1927
1265.	114-1	AMPHI	1934-1946
1266.	114-1	AMPHI	1950-1955
1267.	114-1	AMPHI	1957-1964
1268.	114-1	Antigenic Index	1-6
1269.	114-1	Antigenic Index	10-16
1270.	114-1	Antigenic Index	23-37
1271.	114-1	Antigenic Index	41-55
1272.	114-1	Antigenic Index	75-85
1273.	114-1	Antigenic Index	91-97
1274.	114-1	Antigenic Index	102-140
1275.	114-1	Antigenic Index	147-156
1276.	114-1	Antigenic Index	161-168
1277.	114-1	Antigenic Index	172-174
1278.	114-1	Antigenic Index	181-189
1279.	114-1	Antigenic Index	196-203
1280.	114-1	Antigenic Index	208-213
1281.	114-1	Antigenic Index	220-229
1282.	114-1	Antigenic Index	242-248
1283.	114-1	Antigenic Index	251-266
1284.	114-1	Antigenic Index	268-276
1285.	114-1	Antigenic Index	295-307
1286.	114-1	Antigenic Index	309-312
1287.	114-1	Antigenic Index	318-340
1288.	114-1	Antigenic Index	345-351
1289.	114-1	Antigenic Index	357-366
1290.	114-1	Antigenic Index	371-381
1291.	114-1	Antigenic Index	385-392
1292.	114-1	Antigenic Index	404-417
1293.	114-1	Antigenic Index	419-432
1294.	114-1	Antigenic Index	440-456
1295.	114-1	Antigenic Index	464-468
1296.	114-1	Antigenic Index	473-480
1297.	114-1	Antigenic Index	482-488
1298.	114-1	Antigenic Index	496-511

1299.	114-1	Antigenic Index	515-530
1300.	114-1	Antigenic Index	535-549
1301.	114-1	Antigenic Index	555-560
1302.	114-1	Antigenic Index	564-582
1303.	114-1	Antigenic Index	588-596
1304.	114-1	Antigenic Index	602-615
1305.	114-1	Antigenic Index	617-620
1306.	114-1	Antigenic Index	622-624
1307.	114-1	Antigenic Index	628-632
1308.	114-1	Antigenic Index	637-640
1309.	114-1	Antigenic Index	647-654
1310.	114-1	Antigenic Index	660-666
1311.	114-1	Antigenic Index	668-688
1312.	114-1	Antigenic Index	696-725
1313.	114-1	Antigenic Index	730-733
1314.	114-1	Antigenic Index	738-755
1315.	114-1	Antigenic Index	760-766
1316.	114-1	Antigenic Index	779-783
1317.	114-1	Antigenic Index	786-799
1318.	114-1	Antigenic Index	807-809
1319.	114-1	Antigenic Index	811-819
1320.	114-1	Antigenic Index	831-839
1321.	114-1	Antigenic Index	845-857
1322.	114-1	Antigenic Index	860-862
1323.	114-1	Antigenic Index	864-868
1324.	114-1	Antigenic Index	872-879
1325.	114-1	Antigenic Index	883-891
1326.	114-1	Antigenic Index	893-903
1327.	114-1	Antigenic Index	908-916
1328.	114-1	Antigenic Index	919-936
1329.	114-1	Antigenic Index	941-947
1330.	114-1	Antigenic Index	950-956
1331.	114-1	Antigenic Index	959-976
1332.	114-1	Antigenic Index	979-991
1333.	114-1	Antigenic Index	993-1000
1334.	114-1	Antigenic Index	1007-1022
1335.	114-1	Antigenic Index	1041-1053
1336.	114-1	Antigenic Index	1062-1068
1337.	114-1	Antigenic Index	1075-1108
1338.	114-1	Antigenic Index	1115-1121
1339.	114-1	Antigenic Index	1126-1145
1340.	114-1	Antigenic Index	1148-1152
1341.	114-1	Antigenic Index	1156-1178
1342.	114-1	Antigenic Index	1195-1206
1343.	114-1	Antigenic Index	1208-1212

1344.	114-1	Antigenic Index	1217-1243
1345.	114-1	Antigenic Index	1246-1263
1346.	114-1	Antigenic Index	1271-1282
1347.	114-1	Antigenic Index	1284-1288
1348.	114-1	Antigenic Index	1292-1295
1349.	114-1	Antigenic Index	1299-1307
1350.	114-1	Antigenic Index	1318-1328
1351.	114-1	Antigenic Index	1330-1340
1352.	114-1	Antigenic Index	1344-1359
1353.	114-1	Antigenic Index	1367-1384
1354.	114-1	Antigenic Index	1395-1399
1355.	114-1	Antigenic Index	1405-1417
1356.	114-1	Antigenic Index	1445-1449
1357.	114-1	Antigenic Index	1491-1510
1358.	114-1	Antigenic Index	1526-1529
1359.	114-1	Antigenic Index	1532-1548
1360.	114-1	Antigenic Index	1552-1556
1361.	114-1	Antigenic Index	1560-1562
1362.	114-1	Antigenic Index	1573-1583
1363.	114-1	Antigenic Index	1594-1611
1364.	114-1	Antigenic Index	1627-1635
1365.	114-1	Antigenic Index	1643-1645
1366.	114-1	Antigenic Index	1647-1665
1367.	114-1	Antigenic Index	1680-1686
1368.	114-1	Antigenic Index	1700-1722
1369.	114-1	Antigenic Index	1724-1726
1370.	114-1	Antigenic Index	1739-1746
1371.	114-1	Antigenic Index	1752-1757
1372.	114-1	Antigenic Index	1780-1783
1373.	114-1	Antigenic Index	1791-1795
1374.	114-1	Antigenic Index	1804-1808
1375.	114-1	Antigenic Index	1829-1835
1376.	114-1	Antigenic Index	1841-1859
1377.	114-1	Antigenic Index	1867-1886
1378.	114-1	Antigenic Index	1897-1903
1379.	114-1	Antigenic Index	1908-1912
1380.	114-1	Antigenic Index	1917-1922
1381.	114-1	Antigenic Index	1926-1934
1382.	114-1	Antigenic Index	1938-1945
1383.	114-1	Antigenic Index	1947-1957
1384.	114-1	Antigenic Index	1961-1968
1385.	114-1	Antigenic Index	1974-1978
1386.	114-1	Hydrophilicity	4-6
1387.	114-1	Hydrophilicity	12-15
1388.	114-1	Hydrophilicity	23-34

1389.	114-1	Hydrophilicity	43-55
1390.	114-1	Hydrophilicity	76-85
1391.	114-1	Hydrophilicity	104-110
1392.	114-1	Hydrophilicity	118-123
1393.	114-1	Hydrophilicity	127-132
1394.	114-1	Hydrophilicity	147-154
1395.	114-1	Hydrophilicity	163-167
1396.	114-1	Hydrophilicity	185-187
1397.	114-1	Hydrophilicity	197-203
1398.	114-1	Hydrophilicity	208-211
1399.	114-1	Hydrophilicity	221-227
1400.	114-1	Hydrophilicity	243-245
1401.	114-1	Hydrophilicity	253-261
1402.	114-1	Hydrophilicity	263-266
1403.	114-1	Hydrophilicity	270-272
1404.	114-1	Hydrophilicity	295-301
1405.	114-1	Hydrophilicity	309-312
1406.	114-1	Hydrophilicity	320-328
1407.	114-1	Hydrophilicity	332-337
1408.	114-1	Hydrophilicity	345-351
1409.	114-1	Hydrophilicity	360-366
1410.	114-1	Hydrophilicity	371-378
1411.	114-1	Hydrophilicity	387-392
1412.	114-1	Hydrophilicity	404-415
1413.	114-1	Hydrophilicity	419-432
1414.	114-1	Hydrophilicity	441-450
1415.	114-1	Hydrophilicity	452-456
1416.	114-1	Hydrophilicity	473-480
1417.	114-1	Hydrophilicity	482-485
1418.	114-1	Hydrophilicity	496-500
1419.	114-1	Hydrophilicity	504-509
1420.	114-1	Hydrophilicity	515-520
1421.	114-1	Hydrophilicity	536-549
1422.	114-1	Hydrophilicity	555-560
1423.	114-1	Hydrophilicity	565-568
1424.	114-1	Hydrophilicity	570-579
1425.	114-1	Hydrophilicity	589-594
1426.	114-1	Hydrophilicity	602-604
1427.	114-1	Hydrophilicity	609-615
1428.	114-1	Hydrophilicity	617-620
1429.	114-1	Hydrophilicity	660-666
1430.	114-1	Hydrophilicity	668-680
1431.	114-1	Hydrophilicity	684-686
1432.	114-1	Hydrophilicity	699-708
1433.	114-1	Hydrophilicity	715-725

1434.	114-1	Hydrophilicity	730-733
1435.	114-1	Hydrophilicity	738-744
1436.	114-1	Hydrophilicity	746-754
1437.	114-1	Hydrophilicity	760-766
1438.	114-1	Hydrophilicity	789-793
1439.	114-1	Hydrophilicity	816-818
1440.	114-1	Hydrophilicity	831-836
1441.	114-1	Hydrophilicity	845-857
1442.	114-1	Hydrophilicity	860-862
1443.	114-1	Hydrophilicity	864-866
1444.	114-1	Hydrophilicity	873-879
1445.	114-1	Hydrophilicity	883-885
1446.	114-1	Hydrophilicity	887-889
1447.	114-1	Hydrophilicity	896-899
1448.	114-1	Hydrophilicity	908-916
1449.	114-1	Hydrophilicity	919-932
1450.	114-1	Hydrophilicity	941-947
1451.	114-1	Hydrophilicity	962-975
1452.	114-1	Hydrophilicity	979-989
1453.	114-1	Hydrophilicity	993-1000
1454.	114-1	Hydrophilicity	1007-1022
1455.	114-1	Hydrophilicity	1041-1043
1456.	114-1	Hydrophilicity	1045-1053
1457.	114-1	Hydrophilicity	1062-1068
1458.	114-1	Hydrophilicity	1075-1078
1459.	114-1	Hydrophilicity	1080-1087
1460.	114-1	Hydrophilicity	1089-1104
1461.	114-1	Hydrophilicity	1115-1121
1462.	114-1	Hydrophilicity	1126-1141
1463.	114-1	Hydrophilicity	1143-1145
1464.	114-1	Hydrophilicity	1148-1151
1465.	114-1	Hydrophilicity	1157-1178
1466.	114-1	Hydrophilicity	1197-1203
1467.	114-1	Hydrophilicity	1217-1243
1468.	114-1	Hydrophilicity	1246-1263
1469.	114-1	Hydrophilicity	1271-1273
1470.	114-1	Hydrophilicity	1275-1277
1471.	114-1	Hydrophilicity	1284-1288
1472.	114-1	Hydrophilicity	1299-1307
1473.	114-1	Hydrophilicity	1318-1326
1474.	114-1	Hydrophilicity	1334-1340
1475.	114-1	Hydrophilicity	1350-1355
1476.	114-1	Hydrophilicity	1357-1359
1477.	114-1	Hydrophilicity	1367-1384
1478.	114-1	Hydrophilicity	1407-1417

1479.	114-1	Hydrophilicity	1491-1510
1480.	114-1	Hydrophilicity	1534-1540
1481.	114-1	Hydrophilicity	1576-1583
1482.	114-1	Hydrophilicity	1595-1607
1483.	114-1	Hydrophilicity	1629-1635
1484.	114-1	Hydrophilicity	1643-1645
1485.	114-1	Hydrophilicity	1649-1665
1486.	114-1	Hydrophilicity	1682-1686
1487.	114-1	Hydrophilicity	1704-1722
1488.	114-1	Hydrophilicity	1724-1726
1489.	114-1	Hydrophilicity	1740-1746
1490.	114-1	Hydrophilicity	1804-1806
1491.	114-1	Hydrophilicity	1829-1835
1492.	114-1	Hydrophilicity	1842-1855
1493.	114-1	Hydrophilicity	1876-1879
1494.	114-1	Hydrophilicity	1898-1900
1495.	114-1	Hydrophilicity	1910-1912
1496.	114-1	Hydrophilicity	1920-1922
1497.	114-1	Hydrophilicity	1928-1930
1498.	114-1	Hydrophilicity	1938-1940
1499.	114-1	Hydrophilicity	1948-1954
1500.	114-1	Hydrophilicity	1962-1967
1501.	114a	AMPHI	45-54
1502.	114a	AMPHI	154-160
1503.	114a	AMPHI	182-190
1504.	114a	AMPHI	224-226
1505.	114a	AMPHI	229-233
1506.	114a	AMPHI	285-287
1507.	114a	AMPHI	303-310
1508.	114a	AMPHI	321-332
1509.	114a	AMPHI	348-350
1510.	114a	AMPHI	392-398
1511.	114a	AMPHI	414-416
1512.	114a	AMPHI	478-486
1513.	114a	AMPHI	506-509
1514.	114a	AMPHI	525-529
1515.	114a	AMPHI	565-567
1516.	114a	AMPHI	614-621
1517.	114a	AMPHI	631-635
1518.	114a	AMPHI	770-774
1519.	114a	AMPHI	811-813
1520.	114a	AMPHI	847-849
1521.	114a	AMPHI	851-853
1522.	114a	AMPHI	875-879
1523.	114a	AMPHI	951-959

1524.	114a	AMPHI	975-981
1525.	114a	AMPHI	1034-1036
1526.	114a	AMPHI	1048-1051
1527.	114a	AMPHI	1073-1081
1528.	114a	AMPHI	1086-1090
1529.	114a	AMPHI	1095-1102
1530.	114a	AMPHI	1111-1115
1531.	114a	AMPHI	1163-1166
1532.	114a	AMPHI	1275-1281
1533.	114a	AMPHI	1312-1317
1534.	114a	AMPHI	1338-1347
1535.	114a	AMPHI	1349-1355
1536.	114a	AMPHI	1357-1365
1537.	114a	AMPHI	1376-1398
1538.	114a	AMPHI	1418-1420
1539.	114a	AMPHI	1455-1460
1540.	114a	AMPHI	1472-1484
1541.	114a	AMPHI	1497-1505
1542.	114a	AMPHI	1507-1512
1543.	114a	Antigenic Index	1-6
1544.	114a	Antigenic Index	10-16
1545.	114a	Antigenic Index	23-37
1546.	114a	Antigenic Index	41-55
1547.	114a	Antigenic Index	75-85
1548.	114a	Antigenic Index	91-97
1549.	114a	Antigenic Index	102-137
1550.	114a	Antigenic Index	147-156
1551.	114a	Antigenic Index	161-168
1552.	114a	Antigenic Index	172-174
1553.	114a	Antigenic Index	181-189
1554.	114a	Antigenic Index	196-203
1555.	114a	Antigenic Index	208-213
1556.	114a	Antigenic Index	220-229
1557.	114a	Antigenic Index	242-248
1558.	114a	Antigenic Index	251-266
1559.	114a	Antigenic Index	268-276
1560.	114a	Antigenic Index	295-307
1561.	114a	Antigenic Index	309-312
1562.	114a	Antigenic Index	318-340
1563.	114a	Antigenic Index	345-352
1564.	114a	Antigenic Index	357-366
1565.	114a	Antigenic Index	371-381
1566.	114a	Antigenic Index	385-392
1567.	114a	Antigenic Index	404-427
1568.	114a	Antigenic Index	429-434

1569.	114a	Antigenic Index	440-456
1570.	114a	Antigenic Index	465-468
1571.	114a	Antigenic Index	473-494
1572.	114a	Antigenic Index	496-510
1573.	114a	Antigenic Index	515-530
1574.	114a	Antigenic Index	535-549
1575.	114a	Antigenic Index	555-560
1576.	114a	Antigenic Index	564-578
1577.	114a	Antigenic Index	588-596
1578.	114a	Antigenic Index	602-615
1579.	114a	Antigenic Index	617-620
1580.	114a	Antigenic Index	622-624
1581.	114a	Antigenic Index	628-632
1582.	114a	Antigenic Index	637-640
1583.	114a	Antigenic Index	647-654
1584.	114a	Antigenic Index	660-666
1585.	114a	Antigenic Index	668-688
1586.	114a	Antigenic Index	697-725
1587.	114a	Antigenic Index	730-733
1588.	114a	Antigenic Index	738-755
1589.	114a	Antigenic Index	760-766
1590.	114a	Antigenic Index	779-783
1591.	114a	Antigenic Index	786-799
1592.	114a	Antigenic Index	806-809
1593.	114a	Antigenic Index	811-819
1594.	114a	Antigenic Index	831-839
1595.	114a	Antigenic Index	845-857
1596.	114a	Antigenic Index	860-862
1597.	114a	Antigenic Index	864-868
1598.	114a	Antigenic Index	872-879
1599.	114a	Antigenic Index	883-891
1600.	114a	Antigenic Index	893-902
1601.	114a	Antigenic Index	908-916
1602.	114a	Antigenic Index	923-936
1603.	114a	Antigenic Index	941-947
1604.	114a	Antigenic Index	950-956
1605.	114a	Antigenic Index	959-976
1606.	114a	Antigenic Index	979-989
1607.	114a	Antigenic Index	993-1000
1608.	114a	Antigenic Index	1007-1022
1609.	114a	Antigenic Index	1041-1053
1610.	114a	Antigenic Index	1062-1068
1611.	114a	Antigenic Index	1075-1108
1612.	114a	Antigenic Index	1115-1121
1613.	114a	Antigenic Index	1126-1145

1614.	114a	Antigenic Index	1148-1152
1615.	114a	Antigenic Index	1157-1176
1616.	114a	Antigenic Index	1195-1206
1617.	114a	Antigenic Index	1208-1212
1618.	114a	Antigenic Index	1224-1243
1619.	114a	Antigenic Index	1247-1263
1620.	114a	Antigenic Index	1271-1282
1621.	114a	Antigenic Index	1284-1288
1622.	114a	Antigenic Index	1292-1295
1623.	114a	Antigenic Index	1299-1307
1624.	114a	Antigenic Index	1318-1328
1625.	114a	Antigenic Index	1330-1340
1626.	114a	Antigenic Index	1344-1359
1627.	114a	Antigenic Index	1367-1384
1628.	114a	Antigenic Index	1396-1399
1629.	114a	Antigenic Index	1405-1417
1630.	114a	Antigenic Index	1434-1436
1631.	114a	Antigenic Index	1449-1451
1632.	114a	Antigenic Index	1468-1487
1633.	114a	Antigenic Index	1498-1503
1634.	114a	Antigenic Index	1509-1515
1635.	114a	Antigenic Index	1525-1532
1636.	114a	Hydrophilicity	4-6
1637.	114a	Hydrophilicity	12-15
1638.	114a	Hydrophilicity	23-34
1639.	114a	Hydrophilicity	43-55
1640.	114a	Hydrophilicity	75-85
1641.	114a	Hydrophilicity	104-110
1642.	114a	Hydrophilicity	118-123
1643.	114a	Hydrophilicity	127-132
1644.	114a	Hydrophilicity	147-154
1645.	114a	Hydrophilicity	163-167
1646.	114a	Hydrophilicity	185-187
1647.	114a	Hydrophilicity	197-203
1648.	114a	Hydrophilicity	208-211
1649.	114a	Hydrophilicity	221-227
1650.	114a	Hydrophilicity	243-245
1651.	114a	Hydrophilicity	253-261
1652.	114a	Hydrophilicity	263-266
1653.	114a	Hydrophilicity	270-272
1654.	114a	Hydrophilicity	295-301
1655.	114a	Hydrophilicity	309-312
1656.	114a	Hydrophilicity	320-328
1657.	114a	Hydrophilicity	332-337
1658.	114a	Hydrophilicity	345-351

1659.	114a	Hydrophilicity	360-366
1660.	114a	Hydrophilicity	371-378
1661.	114a	Hydrophilicity	387-392
1662.	114a	Hydrophilicity	404-417
1663.	114a	Hydrophilicity	421-423
1664.	114a	Hydrophilicity	425-427
1665.	114a	Hydrophilicity	442-456
1666.	114a	Hydrophilicity	473-488
1667.	114a	Hydrophilicity	499-509
1668.	114a	Hydrophilicity	515-520
1669.	114a	Hydrophilicity	536-549
1670.	114a	Hydrophilicity	555-560
1671.	114a	Hydrophilicity	565-568
1672.	114a	Hydrophilicity	570-578
1673.	114a	Hydrophilicity	589-594
1674.	114a	Hydrophilicity	602-604
1675.	114a	Hydrophilicity	609-615
1676.	114a	Hydrophilicity	617-620
1677.	114a	Hydrophilicity	660-665
1678.	114a	Hydrophilicity	668-680
1679.	114a	Hydrophilicity	684-686
1680.	114a	Hydrophilicity	699-708
1681.	114a	Hydrophilicity	715-725
1682.	114a	Hydrophilicity	730-733
1683.	114a	Hydrophilicity	738-744
1684.	114a	Hydrophilicity	746-754
1685.	114a	Hydrophilicity	760-766
1686.	114a	Hydrophilicity	789-793
1687.	114a	Hydrophilicity	816-818
1688.	114a	Hydrophilicity	831-836
1689.	114a	Hydrophilicity	845-857
1690.	114a	Hydrophilicity	860-862
1691.	114a	Hydrophilicity	864-866
1692.	114a	Hydrophilicity	873-879
1693.	114a	Hydrophilicity	883-885
1694.	114a	Hydrophilicity	887-889
1695.	114a	Hydrophilicity	896-899
1696.	114a	Hydrophilicity	908-916
1697.	114a	Hydrophilicity	923-932
1698.	114a	Hydrophilicity	941-947
1699.	114a	Hydrophilicity	961-975
1700.	114a	Hydrophilicity	979-989
1701.	114a	Hydrophilicity	993-1000
1702.	114a	Hydrophilicity	1007-1022
1703.	114a	Hydrophilicity	1041-1043

1704.	114a	Hydrophilicity	1045-1053
1705.	114a	Hydrophilicity	1062-1068
1706.	114a	Hydrophilicity	1075-1078
1707.	114a	Hydrophilicity	1080-1087
1708.	114a	Hydrophilicity	1089-1104
1709.	114a	Hydrophilicity	1115-1121
1710.	114a	Hydrophilicity	1126-1141
1711.	114a	Hydrophilicity	1143-1145
1712.	114a	Hydrophilicity	1148-1151
1713.	114a	Hydrophilicity	1158-1171
1714.	114a	Hydrophilicity	1197-1203
1715.	114a	Hydrophilicity	1224-1243
1716.	114a	Hydrophilicity	1251-1263
1717.	114a	Hydrophilicity	1271-1273
1718.	114a	Hydrophilicity	1275-1277
1719.	114a	Hydrophilicity	1284-1288
1720.	114a	Hydrophilicity	1299-1307
1721.	114a	Hydrophilicity	1318-1326
1722.	114a	Hydrophilicity	1334-1340
1723.	114a	Hydrophilicity	1350-1359
1724.	114a	Hydrophilicity	1367-1384
1725.	114a	Hydrophilicity	1407-1417
1726.	114a	Hydrophilicity	1449-1451
1727.	114a	Hydrophilicity	1469-1482
1728.	114a	Hydrophilicity	1484-1486
1729.	114a	Hydrophilicity	1498-1503
1730.	114a	Hydrophilicity	1510-1512
1731.	114a	Hydrophilicity	1527-1532
1732.	124-1	AMPHI	37-43
1733.	124-1	AMPHI	94-96
1734.	124-1	AMPHI	113-115
1735.	124-1	Antigenic Index	20-26
1736.	124-1	Antigenic Index	38-43
1737.	124-1	Antigenic Index	52-55
1738.	124-1	Antigenic Index	62-70
1739.	124-1	Antigenic Index	88-97
1740.	124-1	Antigenic Index	104-114
1741.	124-1	Antigenic Index	123-135
1742.	124-1	Antigenic Index	146-155
1743.	124-1	Hydrophilicity	20-26
1744.	124-1	Hydrophilicity	41-43
1745.	124-1	Hydrophilicity	52-55
1746.	124-1	Hydrophilicity	63-69
1747.	124-1	Hydrophilicity	91-94
1748.	124-1	Hydrophilicity	104-114

1749.	124-1	Hydrophilicity	123-135
1750.	124-1	Hydrophilicity	146-155
1751.	124a	AMPHI	19-21
1752.	124a	AMPHI	23-29
1753.	124a	AMPHI	37-43
1754.	124a	AMPHI	94-96
1755.	124a	Antigenic Index	38-43
1756.	124a	Antigenic Index	52-55
1757.	124a	Antigenic Index	62-70
1758.	124a	Antigenic Index	77-80
1759.	124a	Antigenic Index	90-96
1760.	124a	Antigenic Index	105-115
1761.	124a	Antigenic Index	120-135
1762.	124a	Antigenic Index	145-153
1763.	124a	Hydrophilicity	41-43
1764.	124a	Hydrophilicity	52-55
1765.	124a	Hydrophilicity	63-69
1766.	124a	Hydrophilicity	91-95
1767.	124a	Hydrophilicity	108-115
1768.	124a	Hydrophilicity	120-135
1769.	124a	Hydrophilicity	146-153

It will be understood that the invention is described above by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

TABLE II

The present invention does not include within its scope proteins comprising any of the 45 protein sequences disclosed in WO99/36544. As stated above, if the length of any particular protein sequence disclosed in WO99/36544 is *x* amino acids, the antigenic fragment of the present invention has at most *x-1* amino acids of that protein. For each of the 45 protein sequences given in WO99/36544, the value of *x* is given, for reference, in the following table:

SEQ ID NO:	*x*	SEQ ID NO:	*x*	SEQ ID NO:	*x*	SEQ ID NO:	*X*
2	245	26	571	50	185	74	150
4	591	28	710	52	166	76	255
6	592	30	710	54	326	78	255
8	164	32	62	56	356	80	172
10	321	34	86	58	284	82	242
12	321	36	92	60	1978	84	242
14	124	38	103	62	1532	86	183
16	124	40	85	64	593	88	155
18	173	42	78	66	129	90	153
20	640	44	78	68	319		
22	761	46	219	70	619		
24	111	48	212	72	595		

CLAIMS

1. A fragment of a protein disclosed in WO99/36544, wherein the fragment comprise at least one antigenic determinant.

2. The fragment of claim 1, having a length of 100 amino acids or less.

3. The fragment of claim 1 or claim 2, having a length of 3 amino acids or greater.

4. The fragment of any preceding claim, being one of the 1769 fragments of Table I.

5. A polypeptide having 50% or greater sequence identity to the fragment of any preceding claim.

6. A protein comprising one or more fragment of claim 1, claim 2 or claim 3, with the proviso that the protein is not one of the 45 complete protein sequences disclosed in WO99/36544.

7. An antibody which recognises the fragment according to any one of claims 1 to 4.

8. A protein comprising a peptide sequence, wherein the peptide sequence is recognised by an antibody according to claim 7.

9. Nucleic acid encoding the fragment of claim 1, claim 2 or claim 3, the polypeptide of claim 5, or the protein of claim 8.

10. A composition comprising the fragment of claim 1, claim 2 or claim 3, the polypeptide of claim 5, the protein of claim 8, the antibody of claim 7, and/or the nucleic acid of claim 9, wherein the composition is a vaccine, a diagnostic reagent, or an immunogenic composition.

11. The composition of claim 10 for use as a medicament

12. The use of the fragment of claim 1, claim 2 or claim 3, the polypeptide of claim 5, the protein of claim 8, the antibody of claim 7, and/or the nucleic acid of claim 9, in the manufacture of (i) a medicament for treating or preventing infection due to Neisserial bacteria (ii) a diagnostic reagent for detecting the presence of Neisserial bacteria or of antibodies raised against Neisserial bacteria and/or (iii) a reagent which can raise antibodies against Neisserial bacteria.

13. A method of treating a patient, comprising administering to the patient a therapeutically effective amount of a composition according to claim 10.

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

HALLIBONE, Huw G.
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43 Bloomsbury Square
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PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	07.11.2001
Applicant's or agent's file reference P022260WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB00/01026	13/07/2000	14/07/1999

Applicant
CHIRON SPA

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P022260WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB00/01026	13/07/2000	14/07/1999
International Patent Classification (IPC) or national classification and IPC C12N15/31		
Applicant CHIRON SPA		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 14 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
14/02/2001	07.11.2001
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer Macchia, G Telephone No +31 70 340 4078

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IB00/01026

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-78 as originally filed

Claims, No.:

1-13 as originally filed

Sequence listing part of the description, pages:

1-220, filed with the letter of 18/09/2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 1-13 all in part.

because:

   ☒ the said international application, or the said claims Nos. 13, with respect to industrial applicability and insofar as it refers to subject-matter for which an international search report was established, relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☒ no international search report has been established for the said claims Nos. 1-13 insofar as they concern subject-matter related to inventions 2-15.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

   ☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☐ not complied with for the following reasons:

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☐ all parts.

☒ the parts relating to claims Nos. 1-13, insofar as these claims refer to subject-matter grouped in the first invention as from the separation of the inventions in the International Search Report.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-4, 11, 13
	No:	Claims	5-10, 12
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-13
Industrial applicability (IA)	Yes:	Claims	1-12
	No:	Claims	

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separat sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re It m III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claim 13, insofar as it refers to subject-matter for which an International Search Report was established, relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT.
Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of this claim (Article 34(4)(a)(i) PCT).

Re Item IV

Lack of unity of invention

No required additional search fees were timely paid by the applicant. Therefore, no International Search Report has been established for the subject-matter related to the fragments other than the ones classified under invention 1.
Consequently, no opinion is established for the subject-matter related to the inventions other than invention 1.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1). Reference is made to the following document:

D1: BIEGEL CARSON S.D. et al.: 'Ferric enterobactin binding and utilization by *Neisseria gonorrhoeae*' JOURNAL OF BACTERIOLOGY, vol. 181, no. 9, May 1999, pages 2895-2901, XP002155427.

2.1). Document D1 describes a polypeptide FetB from *Neisseria gonorrhoeae*. The deduced amino acid sequence of FetB was deposited in GenBank under accession number AF115385 and it is 96% identical, over its entire length, to both the amino acid sequences referred to in present application as ORF38-1 and

ORF38a, over their entire length (D1: page 2897). The corresponding DNA sequence was also deposited in GenBank under the same accession number AF115385 (D1: page 2897).
The programs BESTFIT and FASTA of the University of Wisconsin Genetic Computer Group were used for all the sequence homology analyses reported in this communication.
From the amino acid sequence of the protein FetB of D1, it can be noticed that said FetB comprises the fragments identified in pages 38-40 of present application as having the numbers 2-10, 13, 14, 16-23, 25, 26, 29-42, 44, 45, 47-56, 59-65, 67, 69-72, 75-84, 86 and 88-91. Therefore, the polypeptide FetB falls within the scope of the subject-matter of claim 6.

2.2). Document D1 describes two FetB antigenic peptides having the amino acid sequences " SPQNSDPAPQAKGGC " and " CGGEYLKEKNDP " (where the underlined amino acid residues are not part of the FetB protein sequence and function for sulfhydryl coupling). Immunogenic compositions comprising said peptides, the development of polyclonal antisera by means of said peptides and the use of said peptides in the manufacture of a reagent which can raise antibodies against Neisserial bacteria are also described (D1: pages 2896-2897 and figure 3).
The amino acid sequence "SPQNSD**P**APQAK" of said first FetB peptide corresponds to the sequence "SPQNSD**S**APQAK" present in both ORF38-1 and ORF38a, the only difference being the one amino acid residue indicated in bold character.
Analogously, the amino acid sequence "EYLKEKN**DP**" of said second FetB peptide corresponds to the sequence "EYLKEKN**PD**" present in both ORF38-1 and ORF38a, the only difference being the two amino acid residues indicated in bold character. However, also in the FetB amino acid sequence, as deposited in GenBank under accession number AF115385, the corresponding amino acid sequence results to be "EYLKEKN**PD**", thus identical to the one present in ORF38-1 and ORF38a.
In the light of the sequence homology between the peptides of D1 above and the corresponding peptides of present application (indicated as fragment# " 11, 23, 27, 42, 57, 69, 73 and 88 " in pages 38-40 of present application), said **polyclonal** antisera, raised against the peptides of D1 described above, would

cross-react with the polypeptide of present application, thus arriving at the subject-matter of claim 7.

2.3). Claim 8 concerns " a protein comprising a peptide sequence, wherein the peptide sequence is recognized by an antibody according to claim 7 ".
The scope of said claim refers to proteins immunologically cross-reacting with the polypeptide(s) disclosed in present application. In this respect, D1 describes a protein, whose amino acid sequence is 96% identical to both the amino acid sequences referred to in present application as ORF38-1 and ORF38a, as already outlined under point 2.1) above.
In addition to this, D1 describes also antibodies against said protein.
In the light of the above described sequence identity between said FetB protein and the polypeptides having denomination ORF38-1 and ORF38a of present application, the IPEA is of the opinion that the polypeptide of D1 would be recognised by antibodies raised against the polypeptides of present application.

2.4). Having regard to the comments above, the subject-matter of claims **5**, **6**, **7** (for claim 7, insofar as an antibody against a fragment of claims 1-3 and against the fragments of claim 4 indicated in pages 38-40 of present application as fragment# " 11, 23, 27, 42, 57, 69, 73 and 88 " is concerned), **8**, **9** (insofar as claim 9 refers to a nucleic acid encoding the polypeptide of claim 5 or the protein of claim 8), **10** (insofar as claim 10 refers to a composition comprising the polypeptide of claim 5 (wherein the composition is a diagnostic reagent or an immunogenic composition) the protein of claim 8 (wherein the composition is a diagnostic reagent or an immunogenic composition), the antibody of claim 7 (wherein the composition is a diagnostic reagent) or the nucleic acid of claim 9 (wherein the composition is a diagnostic reagent)) and **12** (insofar as claim 12 refers to the use of the polypeptide of claim 5 and of the protein of claim 8 in the manufacture of a diagnostic reagent or a reagent which can raise antibodies; insofar as claim 12 refers to the use of the antibody of claim 7 in the manufacture of a diagnostic reagent; insofar as claim 12 refers to the use of the nucleic acid of claim 9 for the manufacture of a diagnostic reagent) is therefore not novel (Article 33(2) PCT).
In particular, the subject-matter of claims 10 and 12 (insofar as claim 10 refers to the compositions mentioned above and insofar as claim 12 refers to the use of the nucleic acid of claim 9 for the manufacture of a diagnostic reagent) is considered

not to meet the requirements of Article 33(2) PCT because there are no technical features in said claims that allow said subject-matter to be distinguished from the disclosure of document D1.

3). The subject-matter of claims **1-4, 7** (for claim 7, insofar as an antibody against the fragments of claim 4 other than the ones indicated in pages 38-40 of present application as fragment# " 11, 23, 27, 42, 57, 69, 73 and 88 " is concerned), **9** (insofar as claim 9 refers to a nucleic acid encoding the fragments of claims 1-3), **10** (insofar as claim 10 refers to a composition comprising the fragments of claims 1-3 or compositions not mentioned in previous point 2.4), **11, 12** (insofar as claim 12 refers to the use of the fragments of claims 1-3 and to the uses of the polypeptide of claim 5, the protein of claim 8, the antibody of claim 7 and the nucleic acid of claim 9 which are not mentioned under previous point 2.4) and **13** meets the requirements of Article 33(2) PCT because a fragment of a protein as from ORF38-1 or ORF38a, related antibodies (other than the ones against the fragments indicated in pages 38-40 as fragment# " 11, 23, 27, 42, 57, 69, 73 and 88 "), nucleic acids, compositions, uses and methods thereof were not described in the available prior art (document D1).

4.1). Document D1, which is considered to represent the most relevant state of the art, describes a polypeptide FetB from *Neisseria gonorrhoeae* having 96% amino acid sequence identity with both the amino acid sequences referred to in present application as ORF38-1 and ORF38a (D1: page 2897).
Document D1 describes also two FetB antigenic peptides having the amino acid sequences " SPQNSDPAPQAKGGC " and " CGGEYLKEKNDP " (D1: pages 2896-2897), as already commented under previous point 2.1).
The subject-matter of claims 1-4 differs from the disclosure of D1 in that fragments of the polypeptides having denomination ORF38-1 and ORF38a, comprising an antigenic determinant, are concerned.

4.2). The problem to be solved by the present invention may therefore be regarded as the provision of further fragments of a Neisserial polypeptide.

4.3). The solutions proposed in claims 1-3, 4 (insofar as the subject-matter of claim 4 refers to the fragments identified in pages 38-40 of present application as having

the numbers 2-10, 13, 14, 16-23, 25, 26, 29-42, 44, 45, 47-56, 59-65, 67, 69-72, 75-84, 86 and 88-91) of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) for the following reasons: document D1 recites that " two predicted antigenic peptides were identified from the deduced amino acid sequence of *fetB* " (D1: page 2896, right column, last paragraph). This identification, based uniquely on the amino acid sequence of FetB, can be done routinely by means of computer sequence analysis, using algorithms well known to the person skilled in the art, such as for example the algorithms disclosed in page 37 of present application.

This analysis would provide the whole series of FetB peptides, reputed to comprise antigenic determinants. Some of these peptides could also be present in the polypeptides " ORF38-1 " and " ORF38a " of present application, in light of the 96% amino acid sequence identity between these polypeptides and FetB of D1.

As a matter of fact, it should be noted that the fragments identified in pages 38-40 of present application as having the numbers 2-10, 13, 14, 16-23, 25, 26, 29-42, 44, 45, 47-56, 59-65, 67, 69-72, 75-84, 86 and 88-91, which were also found by means of a computer sequence analysis aimed to identify antigenic peptides, are 100% identical in their amino acid sequence to the corresponding fragments in the FetB sequence of D1.

Therefore, in carrying out the identification of antigenic peptides described in D1, the skilled person would inevitably arrive at the identification of fragments which are also embraced by the terms of claims 1-4.

Once identified said fragments, the obtainment of a nucleic acid encoding said fragments, or of antibodies against said fragments should be considered as standard practices which the person skilled in the art is very well aware of.

4.4). In addition to this, it should be noted that present application does not provide any scientific evidence convincingly supporting the presence of antigenic determinants in the fragments disclosed.

As a matter of fact, the fragments described in pages 38-40 of present application were uniquely deduced by means of the algorithms " AMPHI ", " Antigenic Index " and " Hydrophilicity ", as disclosed in page 37 of present application, said algorithms being very well known to the person skilled in the art.

Since present application does not provide any experimental support concerning the antigenicity of any of the fragments disclosed, it should be concluded that the

presence of antigenic determinants in the fragments disclosed in present application and their use is purely speculative.
In the lack of any experimental data supporting the assumption that the fragments concerned comprise an antigenic determinant, no function of said fragments can be envisaged and the problem to be solved is reduced to the problem of provision of new peptide fragments.
This problem is " minimalist " and not technically meaningful.
The solution is a non-inventive selection from a host of polypeptides.

4.5). Having regard to the comments above, it should be concluded that the subject-matter of claims **1-4** does not involve an inventive step (Article 33(3) PCT).
Analogously, the subject-matter of claims **7** and **9-13,** referring to antibodies, compositions comprising a protein comprising said fragments, and uses and methods thereof, also does not involve an inventive step (Article 33(3) PCT).

5.1). In view of the lack of determined antigenic activity of the fragments of present application, and in view of their speculative uses, the industrial applicability does not appear to be plausible (Article 33(4) PCT).
However, with regard to the different laws in the contracting states, no objection is raised for claims 1-10 on this issue.

5.2). For the assessment of the present claims 11, 12 and 13 (insofar as the subject-matter of claim 12 refers to the use of the products concerned in the manufacture of a medicament and insofar as claims 11-13 refer to subject-matter for which an International Search Report was established) on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

R Item VI

Certain documents cited

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO 99/36544	22 July 1999	14 January 1999	14 January 1998 1 September 1998 1 September 1998

Re Item VIII

Certain observations on the international application

1). The following remarks are done with respect to Art. 6, Rule 6 PCT.

2.1). Claim 1 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined.
The following statement " the fragment comprises at least one antigenic determinant " does not enable the skilled person to determine which technical features are necessary for the fragments, to perform the stated function of being antigenic.

2.2). The subject-matter of claims 1, 4 and 6 does not meet the requirement of Rule 6.2 PCT. In fact, in this case it is not " absolutely necessary " to define the fragments concerned by reference to another application or to Table I, because said fragments can be better defined by means of their technical features (see also PCT International Preliminary Examination Guidelines, III-4.10).

3). Present claims 1-3 relate to an extremely large number of possible fragments. In fact, having regard also to the description on pages 1-2, the subject-matter of claim 1 embraces theoretically any fragment of the polypeptide concerned, comprising at least one antigenic determinant, the subject-matter of claim 2 embraces theoretically any fragment of the polypeptide concerned, comprising at least one antigenic determinant and having a length of 100 amino acids or less.

Analogously, the subject-matter of claim 3 embraces theoretically any fragment of the polypeptide concerned, comprising at least one antigenic determinant and having at least 3 amino acid residues.
This makes the subject-matter of claims 1-3 extremely broad and unclear (Article 6 PCT).
Moreover, the description of present application provides disclosure only for a certain number of said fragments, i.e. the ones referring to ORF 38-1 and ORF 38a described in pages 38-40 (Article 5 PCT). Concerning these fragments, it should also be remarked that the amino acid sequence of fragments 2, 9, 31, 32, 40, 47, 53, 55, 77, 78 and 86 is not specified in present application, as from the accompanying sequence listing and the IPEA has deduced the amino acid sequence of said fragments only by making reference to the application WO 99/36544.
Therefore, in the light of the comments above and in the light of the objections under points 2.1) and 2.2), it should be concluded that the subject-matter of claims 1-4 does not meet the requirements of Articles 5 and 6 PCT.
The same remark applies also to the subject-matter of claim 5, where the subject-matter concerned is made even broader and more unclear by the term " having 50% or greater sequence identity to the fragment of any preceding claim ".

4). Claim 5 does not meet the requirements of Article 6 PCT because a functional limitation of the polypeptide concerned is missing.
As such, the scope of claim 5 embraces polypeptides which can be totally structurally and functionally unrelated to the polypeptide of present application. Moreover, in the lack of said functional limitation, the homologous polypeptides might not be linked by a single general inventive concept (Rule 13.1 PCT).

5). Claim 8 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The following functional statement: " the capability of being recognised by an antibody according to claim 7 " does not enable the skilled person to determine which technical features are necessary to perform the stated function.

6). The term " vaccine " in claim 10 implies the generation of a protective immune response against a pathogen or a pathogenic condition. However, not all the

antigens are actually able to induce such protective immune response, although all antigens are immunogenic by definition.
Therefore, in the absence of any experimental data supporting the vaccine composition of claim 10, said subject-matter is considered not to be sufficiently disclosed or supported by the description in the sense of Articles 5 and 6 PCT. Same remarks apply to claims 11, 12(i) and 13.

7). The vague and imprecise statement in the description on page 77 implies that the subject-matter for which protection is sought may be different to that defined by the claims, thereby resulting in lack of clarity (Article 6 PCT) when used to interpret them (see also the PCT Guidelines, III-4.3a).

PATENT COOPERATION TREA

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

To:
HALLIBONE, Huw G.
CARPMAELS & RANSFORD
43 Bloomsbury Square
London WC1A 2RA
GRANDE BRETAGNE

Date of mailing *(day/month/year)*: 02.03.01

Applicant's or agent's file reference: P022260WO

IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB 00/01026	13/07/2000	14/07/1999

Applicant: CHIRON SPA

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

   14/02/2001

2. This date of receipt is:

   - [x] the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
   - [ ] the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
   - [ ] the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. [ ] **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

   [ ] *(If applicable)* This notification confirms the information given by telephone, facsimile transmission or in person on: _____

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/

European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk - Netherlands
Tel.: (+31-70) 340-2040, Tx. 31 651 epo nl
Fax: (+31-70) 340-3016

Authorized officer

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EUROPÄISCHES PATENTAMT · EUROPEAN PATENT OFFICE · OFFICE EUROPÉEN DES BREVETS

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

HALLIBONE, Huw G.
CARPMAELS & RANSFORD
43 Bloomsbury Square
London WC1A 2RA
GRANDE BRETAGNE

Fax no: 020-74054166

PCT

WRITTEN OPINION

(PCT Rule 66)

By Fax in advance

Date of mailing *(day/month/year)*	12.06.2001
Applicant's or agent's file reference: P022260WO	**REPLY DUE** **within 1 month(s) and 15 days** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB00/01026	13/07/2000	14/07/1999

International Patent Classification (IPC) or both national classification and IPC

C12N15/31

Applicant

CHIRON SPA

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☒	Certain document cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 14/11/2001.

Name and mailing address of the international preliminary examining authority:

European Patent Office - P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk - Pays Bas
Tel. +31 70 340 - 2040 Tx: 31 651 epo nl
Fax: +31 70 340 - 3016

Authorized officer / Examiner

Macchia, G

Formalities officer (incl. extension of time limits)
Sinanovic, E
Telephone No. +31 70 340 2672

I. Basis of the opinion

1. With regard to the **elements** of the international application (Replacement *sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed"*):

Description, pages:

1-78 as originally filed

Claims, No.:

1-13 as originally filed

Sequence listing part of the description, pages:

1-220, filed with the letter of 18/09/2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

   ☐ the entire international application,

   ☒ claims Nos. 1-13 all in part,

because:

   ☒ the said international application, or the said claims Nos. 13, with respect to industrial applicability and insofar as it refers to subject-matter for which an international search report was established, relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☒ no international search report has been established for the said claims Nos. 1-13 insofar as they concern subject-matter related to inventions 2-15.

2. A written opinion cannot be drawn due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	5, 7-10, 12
Inventive step (IS)	Claims	1-4, 6, 9-13
Industrial applicability (IA)	Claims	

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1). No required additional search fees were timely paid by the applicant. Therefore, no International Search Report has been established for the subject-matter related to the fragments other than the ones classified under invention 1. Consequently, no opinion is established for the subject-matter related to the inventions other than invention 1.

2). Claim 13, insofar as it refers to subject-matter for which an International Search Report was established, relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT.
Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of this claim (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1). Reference is made to the following document:

D1: BIEGEL CARSON S.D. et al.: 'Ferric enterobactin binding and utilization by *Neisseria gonorrhoeae*' JOURNAL OF BACTERIOLOGY, vol. 181, no. 9, May 1999, pages 2895-2901, XP002155427.

2.1). Document D1 describes a polypeptide FetB from *Neisseria gonorrhoeae*. The deduced amino acid sequence of FetB was deposited in GenBank under accession number AF115385 and it is 96% identical to both the amino acid sequences referred to in present application as ORF38-1 and ORF38a (D1: page 2897). The corresponding DNA sequence was also deposited in GenBank under the same accession number AF115385 (D1: page 2897).
Document D1 describes two FetB antigenic peptides having the amino acid sequences " SPQNSDPAPQAKGGC " and " CGGEYLKEKNDP " (where the

underlined amino acid residues are not part of the FetB protein sequence and function for sulfhydryl coupling). Immunogenic compositions comprising said peptides, the development of polyclonal antisera by means of said peptides and the use of said peptides in the manufacture of a reagent which can raise antibodies against Neisserial bacteria are also described (D1: pages 2896-2897 and figure 3).

The amino acid sequence "SPQNSD**P**APQAK" of said first FetB peptide corresponds to the sequence "SPQNSD**S**APQAK" present in ORF38-1 and ORF38a, the only difference being the one amino acid residue indicated in bold character.

Analogously, the amino acid sequence "EYLKEKN**DP**" of said second FetB peptide corresponds to the sequence "EYLKEKN**PD**" present in ORF38-1 and ORF38a, the only difference being the two amino acid residues indicated in bold character. However, also in the FetB amino acid sequence, as deposited in GenBank under accession number AF115385, the corresponding amino acid sequence results to be "EYLKEKN**PD**", thus identical to the one present in ORF38-1 and ORF38a.

In the light of the sequence homology between the peptides of D1 above and the corresponding peptides of present application, said **polyclonal** antisera, raised against the peptides of D1 described above, would cross-react with the polypeptide of present application, thus arriving at the subject-matter of claim 7.

2.2). Claim 8 concerns " a protein comprising a peptide sequence, wherein the peptide sequence is recognized by an antibody according to claim 7 ".

The scope of said claim refers to proteins immunologically cross-reacting with the polypeptide(s) disclosed in present application. In this respect, D1 describes a protein, whose amino acid sequence is 96% identical to both the amino acid sequences referred to in present application as ORF38-1 and ORF38a, as already outlined under point 2.1) above.

In addition to this, D1 describes also antibodies against said protein.

In the light of the above described sequence identity between said FetB protein and the polypeptides having denomination ORF38-1 and ORF38a of present application, the IPEA is of the opinion that the polypeptide of D1, would be recognised by antibodies raised against the polypeptides of present application.

2.3). Having regard to the comments above, the subject-matter of claims 5, 7, 8, 9 (insofar as claim 9 refers to a nucleic acid encoding the polypeptide of claim 5 or the protein of claim 8), 10 (insofar as claim 10 refers to a composition comprising the polypeptide of claim 5, the protein of claim 8, the antibody of claim 7 or the nucleic acid of claim 9), and 12 (insofar as claim 12 refers to the use of the polypeptide of claim 5, the protein of claim 8, the antibody of claim 7 or the nucleic acid of claim 9) is therefore not novel (Article 33(2) PCT).

3.1). Document D1, which is considered to represent the most relevant state of the art, describes a polypeptide FetB from *Neisseria gonorrhoeae* having 96% amino acid sequence identity with both the amino acid sequences referred to in present application as ORF38-1 and ORF38a (D1: page 2897).
Document D1 describes also two FetB antigenic peptides having the amino acid sequences " SPQNSDPAPQAKGGC " and " CGGEYLKEKNDP " (D1: pages 2896-2897), as already commented under previous point 2.1).
The subject-matter of claim 1 differs from the disclosure of D1 in that fragments of the polypeptides having denomination ORF38-1 and ORF38a, comprising an antigenic determinant, are concerned.

3.2). The problem to be solved by the present invention may therefore be regarded as the provision of further fragments of a Neisserial polypeptide.

3.3). The solutions proposed in claims 1-4 of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) for the following reasons.
Document D1 recites that " two predicted antigenic peptides were identified from the deduced amino acid sequence of *fetB* " (D1: page 2896, right column, last paragraph). This identification, based uniquely on the amino acid sequence of FetB, can be done routinely by means of computer sequence analysis, using algorithms well known to the person skilled in the art, such as for example the algorythms disclosed in page 37 of present application.
This analysis would provide the whole series of FetB peptides, reputed to comprise antigenic determinants. Some of these peptides could also be present in the polypeptides " ORF38-1 " and " ORF38a " of present application, in light of the 96% amino acid sequence identity between these polypeptides and FetB of D1.

As a matter of fact, it should be noted that the fragments identified in pages 38-40 of present application as having the numbers 3-8, 10, 13, 14, 16-23, 25, 26, 29, 30, 33-39, 41, 42, 44, 45, 48-52, 54, 56, 59-65, 67, 69-72, 75, 76, 79-84 and 88-91, which were also found by means of a computer sequence analysis aimed to identify antigenic peptides, are 100% identical in their amino acid sequence to the corresponding fragments in the FetB sequence of D1.
Therefore, in carrying out the identification of antigenic peptides described in D1, the skilled person would inevitably arrive at the identification of fragments which are also embraced by the terms of claims 1-4 and of a protein comprising said fragments, which is also embraced by the terms of claim 6.

3.4). In addition to this, it should be noted that present application does not provide any scientific evidence convincingly supporting the presence of antigenic determinants in the fragments disclosed.
As a matter of fact, the fragments described in pages 38-40 of present application were uniquely deduced by means of the algorithms " AMPHI ", " Antigenic Index " and " Hydrophilicity ", as disclosed in page 37 of present application, said algorithms being very well known to the person skilled in the art.
Therefore, in the lack of any experimental data supporting the antigenicity of any of the fragments disclosed, it should be concluded that the presence of antigenic determinants in the fragments disclosed in present application is purely speculative.
Analogously, since present application does not provide any experimental support to the assumption that the fragments concerned comprise an antigenic determinant, its therapeutic role is also purely speculative.

3.5). Having regard to the comments above and also to the fact that a product is not rendered novel merely by the fact that it is produced by a possibly new process, it should be concluded that the subject-matter of claims 1-4, 6, 9 (insofar as claim 9 refers to a nucleic acid encoding the fragments of claim 1-3), 10 (insofar as claim 10 refers to a composition comprising the fragment of claims 1-3), 11, 12 (insofar as claim 12 refers to the fragments of claims 1-3) and 13 does not involve an inventive step (Article 33(3) PCT).

4). For the assessment of the present claim 13, insofar as it refers to subject-matter

for which an International Search Report was established, on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claim. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

5.1). The applicant is requested to file amendments by way of replacement pages in the manner stipulated by Rule 66.8(a) PCT. In particular, fair copies of the amendments should be filed preferably in triplicate.
In order to facilitate the examination of the conformity of the amended application with the requirements of Article 34(2)(b) PCT, the applicant is requested to clearly identify the amendments carried out, irrespective of whether they concern amendments by addition, replacement or deletion, and to indicate the passages of the application as filed on which these amendments are based.
If the applicant regards it as appropriate these indications could be submitted in handwritten form on a copy of the relevant parts of the application as filed.

5.2). Moreover, the applicant's attention is drawn to the fact that, as a consequence of Rule 66.8(a) PCT the examiner is not permitted to carry out any amendments under the PCT procedure, however minor these may be.

Re Item VI
Certain documents cited

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO 99/36544	22 July 1999	14 January 1999	14 January 1998 1 September 1998 9 October 1998

Re Item VIII

Certain observations on the international application

1). The following remarks are done with respect to Art. 6, Rule 6 PCT.

2.1). Claim 1 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined.
The following statement " the fragment comprises at least one antigenic determinant " does not enable the skilled person to determine which technical features are necessary for the fragments, to perform the stated function of being antigenic.

2.2). The subject-matter of claims 1, 4 and 6 does not meet the requirement of Rule 6.2 PCT. In fact, in this case it is not " absolutely necessary " to define the fragments concerned by reference to another application or to Table I, because said fragments can be better defined by means of their technical features (see also PCT International Preliminary Examination Guidelines, III-4.10).

3). Present claims 1-3 relate to an extremely large number of possible fragments. In fact, having regard also to the description on pages 1-2, the subject-matter of claim 1 embraces theoretically any fragment of the polypeptide concerned, comprising at least one antigenic determinant, the subject-matter of claim 2 embraces theoretically any fragment of the polypeptide concerned, comprising at least one antigenic determinant and having a length of 100 amino acids or less. Analogously, the subject-matter of claim 3 embraces theoretically any fragment of the polypeptide concerned, comprising at least one antigenic determinant and having at least 3 amino acid residues.
This makes the subject-matter of claims 1-3 extremely broad and unclear (Article 6 PCT).
Moreover, the description of present application provides disclosure only for a certain number of said fragments, i.e. the ones referring to ORF 38-1 and ORF 38a described in pages 38-40 (Article 5 PCT). concerning these fragments, it should also be remarked that the amino acid sequence of fragments 2, 9, 31, 32, 40, 47, 53, 55, 77, 78 and 86 is not specified in present application.
Therefore, in the light of the comments above and in the light of the objections

under points 2.1) and 2.2), it should be concluded that the subject-matter of claims 1-3 does not meet the requirements of Articles 5 and 6 PCT.
The same remark applies also to the subject-matter of claim 5, where the subject-matter concerned is made even broader and more unclear by the term " having 50% or greater sequence identity to the fragment of any preceding claim ".

4). In claim 5, a functional limitation of the polypeptide concerned should be given. In fact the scope of claim 5 embraces polypeptides which can be totally structurally and functionally unrelated to the polypeptide of present application. Failing said functional limitation, the homologous polypeptides might not be linked by a single general inventive concept (Rule 13.1 PCT).

5). Claim 8 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The following functional statement: " the capability of being recognised by an antibody according to claim 7 " does not enable the skilled person to determine which technical features are necessary to perform the stated function.

6). The term " vaccine " in claim 10 implies the generation of a protective immune response against a pathogen or a pathogenic condition. However, not all the antigens are actually able to induce such protective immune response, although all antigens are immunogenic by definition.
Therefore, in the absence of any experimental data supporting the vaccine composition of claim 10, said subject-matter is considered not to be sufficiently disclosed or supported by the description in the sense of Articles 5 and 6 PCT.
Same remarks apply to claims 11, 12(i) and 13.

7). The vague and imprecise statement in the description on page 77 implies that the subject-matter for which protection is sought may be different to that defined by the claims, thereby resulting in lack of clarity (Article 6 PCT) when used to interpret them (see also the PCT Guidelines, III-4.3a).

8). In the light of the comments above, no full examination of present application can be carried out until the claims are not drafted so to meet the requirements of Articles 5 and 6 PCT.

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority must be indicated by the applicant on the line below:

IPEA/ _____

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: P022260WO: HGH/CJM

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/IB00/01026	13/07/2000	14/07/1999

Title of invention: ANTIGENIC MENINGOCOCCAL PEPTIDES

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

CHIRON S.p.A
VIA FIORENTINA 1
53100 SIENA
ITALY

Telephone No.:

Facsimile No.:

Teleprinter No.:

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
IT	IT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

MASIGNANI Vega
Chiron S.p.A.
Via Fiorentina 1
53100 Siena
ITALY

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
IT	IT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SCARLATO Vincenzo
Chiron S.p.A.
Via Fiorentina 1
53100 Siena
ITALY

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
IT	IT

[X] Further applicants are indicated on a continuation sheet.

Sheet No. 2

International application No.
PCT/IB00/01026

Continuation of Box No. II APPLICANT(S)

If none of the following sub-boxes is used, this sheet should not be included in the demand.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SCARSELLI Maria
Chiron S.p.A
Via Fiorentina 1
53100 Siena
ITALY

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
IT	IT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

GALEOTTI Cesira
Chiron S.p.A.
Via Fiorentina 1
53100 Siena
ITALY

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
IT	IT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

MORA Mariarosa
Chiron S.p.A
Via Fiorentina 1
53100 Siena
ITALY

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
IT	IT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(that is, country)* of nationality:	State *(that is, country)* of residence:

☐ Further applicants are indicated on another continuation sheet.

International application No.
PCT/IB00/01026

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [X] agent [ ] common representative

and [X] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HALLYBONE, Huw George
CARPMAELS & RANSFORD
43 BLOOMSBURY SQUARE
LONDON WC1A 2RA
UNITED KINGDOM

Telephone No.: 020-7242 8692

Facsimile No.: 020-7405 4166

Teleprinter No.: 267209

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special addr ess to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination to start on the basis of:

[X] the international application as originally filed

the description [ ] as originally filed
[ ] as amended under Article 34

the claims [ ] as originally filed
[ ] as amended under Article 19 (together with any accompanying statement)
[ ] as amended under Article 34

the drawings [ ] as originally filed
[ ] as amended under Article 34

2. [ ] The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. [ ] The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: ENGLISH

[X] which is the language in which the international application was filed.
[ ] which is the language of a translation furnished for the purposes of international search.
[ ] which is the language of publication of the international application.
[ ] which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby **elects all eligible States** *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes **not to elect:**

International application No.
PCT/IB00/01026

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	:	sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. [x] fee calculation sheet
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] nucleotide and or amino acid sequence listing in computer readable form
6. [ ] other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

HALLYBONE, Huw George

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. [ ] The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. [ ] The applicant has been informed accordingly.
4. [ ] The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. [ ] Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

CHAPTER II

PCT

FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

International application No.	PCT/IB00/01026
Applicant's or agent's file reference	P022260WO: HGH
Applicant	CHIRON S.p.A.

For International Preliminary Examining Authority use only

Date stamp of the IPEA

Calculation of prescribed fees

1. Preliminary examination fee	EUR 1533	P
2. Handling fee *(Applicants from certain States are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.)*	EUR 147	H
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box	EUR 1680	TOTAL

Mode of Payment

[X]	authorization to charge deposit account with the IPEA (see below)	[ ]	cash
[ ]	cheque	[ ]	revenue stamps
[ ]	postal money order	[ ]	coupons
[ ]	bank draft	[ ]	other *(specify)*:

Deposit Account Authorization *(this mode of payment may not be available at all IPEAs)*

The IPEA/ _____ [X] is hereby authorized to charge the total fees indicated above to my deposit account.

[X] *(this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

2805.0059	31.01.2001	CARPMAELS & RANSFORD
Deposit Account Number	Date *(day/month/year)*	Signature

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
CARPMAELS & RANSFORD
Attn. HALLIBONE, Huw G.
43 Bloomsbury Square
London WC1A 2RA
UNITED KINGDOM

Applicant's or agent's file reference P022260WO	Date of mailing *(day/month/year)* 09/03/2001
International application No. PCT/IB 00/ 01026	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
Applicant CHIRON SPA	International filing date *(day/month/year)* 13/07/2000

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Mireille Claudepierre

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments and any accompanying statement, under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the time of filing the amendments (and any statement) with the International Bureau, also file with the International Preliminary Examining Authority a copy of such amendments (and of any statement) and, where required, a translation of such amendments for the procedure before that Authority (see Rules 55.3(a) and 62.2, first sentence). For further information, see the Notes to the demand form (PCT/IPEA/401).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
P022260WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IB 00/01026	13/07/2000	14/07/1999
Applicant		
CHIRON SPA		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [X] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. -

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 00/01026

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 13 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

claims 1-13 all partially

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-13 all partially

A fragment of a protein disclosed in WO 99/36544 as ORF38-1, or ORF38a, wherein the fragment is chosen among fragments 1-91 of Table I.

A polypeptide having 50% or greater sequence identity to one of said fragments.
A protein comprising one or more of said fragments.
An antibody which recognises said fragments.
A protein comprising a peptide sequence recognised by said antibody.
Nucleic acid encoding said fragments, polypeptide or protein.
Related compositions and uses.

2. Claims: 1-13 all partially

As invention 1 but concerning fragments 92-296 from ORF39-1 or ORF39a.

3. Claims: 1-13 all partially

As invention 1 but concerning fragments 297-467 from ORF40-1 or ORF40-a.

4. Claims: 1-13 all partially

As invention 1 but concerning fragments 468-655 from ORF41-1 or ORF41-a.

5. Claims: 1-13 all partially

As invention 1 but concerning fragments 656-689 from ORF44-1 or ORF44-a.

6. Claims: 1-13 all partially

As invention 1 but concerning fragments 690-905 from ORF49-1 or ORF49-a.

7. Claims: 1-13 all partially

As invention 1 but concerning fragments 906-940 from ORF51-1 or ORF51-a.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

8. Claims: 1-13 all partially

As invention 1 but concerning fragments 941-951 from ORF52-1.

9. Claims: 1-13 all partially

As invention 1 but concerning fragments 952-965 from ORF69-1 or ORF69-a.

10. Claims: 1-13 all partially

As invention 1 but concerning fragments 966-1003 from ORF77-1 or ORF77-a.

11. Claims: 1-13 all partially

As invention 1 but concerning fragments 1004-1073 from ORF81-1.

12. Claims: 1-13 all partially

As invention 1 but concerning fragments 1074-1101 from ORF82-a.

13. Claims: 1-13 all partially

As invention 1 but concerning fragments 1102-1193 from ORF112-1 or ORF112-a.

14. Claims: 1-13 all partially

As invention 1 but concerning fragments 1194-1731 from ORF114-1 or ORF114-a.

15. Claims: 1-13 all partially

As invention 1 but concerning fragments 1732-1769 from ORF124-1 or ORF124-a.

Int. ational Application No
PCT/IB 00/01026

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C07K14/22 G01N33/53 C12Q1/68 C07K16/12
A61K39/095 A61K39/395 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIEGEL CARSON S.D. ET AL.: "Ferric enterobactin binding and utilization by Neisseria gonorrhoeae" JOURNAL OF BACTERIOLOGY, vol. 181, no. 9, May 1999 (1999-05), pages 2895-2901, XP002155427 page 2896, right-hand column, paragraph 4 -page 2897, left-hand column, paragraph 1 page 2898; figure 3 --- -/--	1-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 2000	09. 03. 01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 99 36544 A (CHIRON S.P.A. (IT) MASIGNANI V; RAPPUOLI R; PIZZA M; SCARLATO; GRANDI) 22 July 1999 (1999-07-22) cited in the application	1-3,5-13
L	L: priority page 3, line 17-20 page 3, line 25 -page 4, line 12 page 48, line 16 -page 50, line 22 page 66 -page 68; example 2	

A	WO 90 06696 A (PRAXIS BIOL INC (US) RIJKINSINST. VOOR VOLKSGEZONDHEID EN MILIEUHYGINE) 28 June 1990 (1990-06-28) page 45 -page 51; example 6 page 68 -page 73; example 13 page 78 -page 86; example 16 page 94 -page 103; claims	

A	CHRISTODOULIDES M. AND HECKELS J.E.: "Immunization with a multiple antigen peptide containing defined B- and T-cell epitopes: production of bactericidal antibodies against group B Neisseria meningitidis" MICROBIOLOGY, vol. 140, no. 11, November 1994 (1994-11), pages 2951-2960, XP000867241 ISSN: 1350-0872 abstract page 2952, right-hand column, paragraph 2	

A	DELVIG A.A. ET AL.: "Immune responses to linear epitopes on the PorB protein of Neisseria meningitidis in patients with systemic meningococcal disease" MICROBIOLOGY, vol. 142, no. 9, September 1996 (1996-09), pages 2491-2498, XP000856354 ISSN: 1350-0872 abstract	

A	ROKBI B. ET AL.: "Variable sequences in a mosaic-like domain of meningococcal tbp2 encode immunoreactive epitopes" FEMS MICROBIOLOGY LETTERS, vol. 132, 1995, pages 277-283, XP000578968 ISSN: 0378-1097 page 282; table 2	

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9936544 A	22-07-1999	AU 1979599 A	02-08-1999
		EP 1047784 A	02-11-2000
WO 9006696 A	28-06-1990	NL 8803111 A	16-07-1990
		NL 8900030 A	01-08-1990
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		AT 120093 T	15-04-1995
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		JP 6503465 T	21-04-1994
		NL 8900036 A	16-07-1990
		NO 305463 B	07-06-1999
		CA 2000735 A	06-07-1990
		CA 2007248 A	06-07-1990
		DE 8901378 U	23-03-1989
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		DK 511389 A	07-07-1990
		EP 0377233 A	11-07-1990
		ES 2029372 T	01-08-1992
		US 5057007 A	15-10-1991
		PT 92807 A,B	31-07-1990

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* P022260WO

Box No. I TITLE OF INVENTION

ANTIGENIC MENINGOCOCCAL PEPTIDES

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

CHIRON SpA
Via Fiorentina 1
53100 Siena
ITALY

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

MASIGNANI Vega
Chiron SpA
Via Fiorentina 1
53100 Siena
ITALY

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HALLYBONE Huw George
CARPMAELS & RANSFORD
43 BLOOMSBURY SQUARE
LONDON WC1A 2RA
GB

Telephone No. +44 20-7242 8692

Facsimile No. +44 20-7405 4166

Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

SCARLATO Vincenzo
Chiron SpA
Via Fiorentina 1
53100 Siena
ITALY

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

SCARSELLI Maria
Chiron SpA
Via Fiorentina 1
53100 Siena
ITALY

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GALEOTTI Cesira
Chiron SpA
Via Fiorentina 1
53100 Siena
ITALY

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

MORA Maria Rosa
Chiron SpA
Via Fiorentina 1
53100 Siena
ITALY

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☑ **AP** **ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **MZ** Mozambique, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **TZ** United Republic of Tanzania, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☑ **EA** **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☑ **EP** **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☑ **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☑ **AE** United Arab Emirates
- ☑ **AG** Antigua and Barbuda
- ☑ **AL** Albania
- ☑ **AM** Armenia
- ☑ **AT** Austria
- ☑ **AU** Australia
- ☑ **AZ** Azerbaijan
- ☑ **BA** Bosnia and Herzegovina
- ☑ **BB** Barbados
- ☑ **BG** Bulgaria
- ☑ **BR** Brazil
- ☑ **BY** Belarus
- ☑ **BZ** Belize
- ☑ **CA** Canada
- ☑ **CH and LI** Switzerland and Liechtenstein
- ☑ **CN** China
- ☑ **CR** Costa Rica
- ☑ **CU** Cuba
- ☑ **CZ** Czech Republic
- ☑ **DE** Germany
- ☑ **DK** Denmark
- ☑ **DM** Dominica
- ☑ **DZ** Algeria
- ☑ **EE** Estonia
- ☑ **ES** Spain
- ☑ **FI** Finland
- ☑ **GB** United Kingdom
- ☑ **GD** Grenada
- ☑ **GE** Georgia
- ☑ **GH** Ghana
- ☑ **GM** Gambia
- ☑ **HR** Croatia
- ☑ **HU** Hungary
- ☑ **ID** Indonesia
- ☑ **IL** Israel
- ☑ **IN** India
- ☑ **IS** Iceland
- ☑ **JP** Japan
- ☑ **KE** Kenya
- ☑ **KG** Kyrgyzstan
- ☑ **KP** Democratic People's Republic of Korea
- ☑ **KR** Republic of Korea
- ☑ **KZ** Kazakhstan
- ☑ **LC** Saint Lucia
- ☑ **LK** Sri Lanka
- ☑ **LR** Liberia
- ☑ **LS** Lesotho
- ☑ **LT** Lithuania
- ☑ **LU** Luxembourg
- ☑ **LV** Latvia
- ☑ **MA** Morocco
- ☑ **MD** Republic of Moldova
- ☑ **MG** Madagascar
- ☑ **MK** The former Yugoslav Republic of Macedonia
- ☑ **MN** Mongolia
- ☑ **MW** Malawi
- ☑ **MX** Mexico
- ☑ **MZ** Mozambique
- ☑ **NO** Norway
- ☑ **NZ** New Zealand
- ☑ **PL** Poland
- ☑ **PT** Portugal
- ☑ **RO** Romania
- ☑ **RU** Russian Federation
- ☑ **SD** Sudan
- ☑ **SE** Sweden
- ☑ **SG** Singapore
- ☑ **SI** Slovenia
- ☑ **SK** Slovakia
- ☑ **SL** Sierra Leone
- ☑ **TJ** Tajikistan
- ☑ **TM** Turkmenistan
- ☑ **TR** Turkey
- ☑ **TT** Trinidad and Tobago
- ☑ **TZ** United Republic of Tanzania
- ☑ **UA** Ukraine
- ☑ **UG** Uganda
- ☑ **US** United States of America
- ☑ **UZ** Uzbekistan
- ☑ **VN** Viet Nam
- ☑ **YU** Yugoslavia
- ☑ **ZA** South Africa
- ☑ **ZW** Zimbabwe

Check-box reserved for designating States which have become party to the PCT after issuance of this sheet:

☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included in the request.*

1. If, in any of the Boxes, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No. ...." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:

(i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;

(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;

(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;

(iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;

(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "continuation" or "continuation-in-part": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;

(vi) if, in Box No. VI, there are more than three earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;

(vii) if, in Box No. VI, the earlier application is an ARIPO application: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed.

2. If, with regard to the precautionary designation statement contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.

3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.

Continuation of Box No. IV

VIELLE DEVAUX, Ian Benedict Peter
AEL, John William Maurice
JONES, Alan John
COLGAN, Stephen James
HOWICK, Nicholas Keith
FISHER, Adrian John
MERCER, Christopher Paul
HALLYBONE, Huw George
JACKSON, Richard Eric
HOWARD, Paul Nicholas
JAMES, Anthony Christopher W.P.

also of CARPMAELS & RANSFORD, 43 Bloomsbury Square, London WC1A 2RA, United Kingdom

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 14th July 1999 (14.07.99)	9916529.2	GB		
item (2)				
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s):

** Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA / EP

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority)*:

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	: 5
description (excluding sequence listing part)	: 78
claims	: 1
abstract	: 1
drawings	: 0
sequence listing part of description	: 0
Total number of sheets	: 85

This international application is **accompanied** by the item(s) marked below:

1. ☑ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

Figure of the drawings which should accompany the abstract: -

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

HALLYBONE Huw George

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET
Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference P022260GB

Applicant
CHIRON SpA et al.

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . £ 55 T

2. SEARCH FEE . . . . . S

International search to be carried out by _____

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains _____ sheets.

first 30 sheets b1

_____ x _____ = b2
remaining sheets additional amount

Add amounts entered at b1 and b2 and enter total at B . . . . B

Designation Fees

The international application contains _____ designations.

_____ x _____ = D
number of designation fees payable *(maximum 8)* amount of designation fee

Add amounts entered at B and D and enter total at I I

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . P

5. TOTAL FEES PAYABLE . . . . . £ 55
Add amounts entered at T, S, I and P, and enter total in the TOTAL box TOTAL

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

☒ authorization to charge deposit account (see below)	☐ bank draft	☐ coupons
☐ cheque	☐ cash	☐ other *(specify):*
☐ postal money order	☐ revenue stamps	

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ GB ☒ is hereby authorized to charge the total fees indicated above to my deposit account.

☐ *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

D00019	13 July 2000	
Deposit Account No.	Date *(day/month/year)*	Signature